Epstein-Barr virus associated diseases; immunotherapy and cytokine gene expression.

Dr Mobin Asghar

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I hereby declare that this thesis has been composed by myself and has neither been presented or accepted in any previous application or degree. This thesis is a record of work carried out by myself except where stated otherwise. All sources of information have been acknowledged by reference in the bibliography

Dr Mobin Asghar
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Abstract
Epstein-Barr virus (EBV) establishes persistent infection in over 90% of adults worldwide. It is associated with a number of human diseases including infectious mononucleosis (IM), nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD) and post-transplant lymphoproliferative disease (PTLD). This thesis reports two separate studies on immunotherapy of EBV positive PTLD and on pathogenesis of EBV related diseases in relation to cytokine gene expression. PTLD is a B cell malignancy seen in 1-10% of solid organ transplant recipients. Severe combined immunodeficient (SCID) mice serve as a murine model for PTLD because subcutaneous (s/c) inoculation of in vitro transformed lymphoblastoid cell lines (LCLs) result in human EBV-positive PTLD like tumours in these animals. The aim of the first study was to use the SCID model to investigate the effect of varying doses of human unmanipulated peripheral blood mononuclear cells (PBMC), polyclonal and EBV peptide-specific cytotoxic T cell lines (CTL) on outgrowth and therapy of autologous LCL tumours. Effector cells were mixed with fixed tumorigenic dose of autologous LCL cells at varying ratios and injected s/c into SCID mice. Unmanipulated PBMC failed to control the outgrowth of LCL tumours. Polyclonal CTLs prevented tumour outgrowth down to the CTL:LCL ratio of 0.25:1 suggesting that outgrowth of EBV-positive tumours is effectively controlled by autologous EBV-specific CTLs. Polyclonal CTLs also improved survival of SCID mice with already established autologous LCL tumours. Positive effect of CTL on survival and tumour mass reduction was greater in animals with small tumour mass at start of therapy as compared to those with larger tumours. Delivery of multiple CTL infusions via the intravenous (I/V) route was more effective in reducing tumour mass than injection of a single fixed CTL dose via the I/V or intra-tumour route. In contrast, only the EBV peptide-specific CTLs with a higher frequency of detectable precursors in the peripheral blood prevented tumour outgrowth below CTL:LCL ratio of 1:1. Peptide-specific CTLs targetting an immunodominant LMP1 epitope also successfully controlled outgrowth of LCL tumours below CTL:LCL ratio of 1:1, suggesting the possible extension of CTL therapy to EBV positive malignancies with restricted EBV gene expression patterns.

The second study investigated the cytokine gene expression pattern of EBV associated diseases. Dysregulation of the cytokine network has been proposed as a cofactor in pathogenesis of EBV related conditions. Higher level of cytokine gene expression has been found in lymphoid tissue of patients with IM as compared to normal individuals and in cases of EBV positive PTLD, HD and NPC as compared to EBV negative cases. In our study we investigated the cytokine profile of a tonsil from an acute IM case, human PBMC induced SCID tumours, PTLD, NPC and HD by non-isotopic in situ hybridisation. We also identified the cell type responsible for cytokine production and its relationship to EBV infection. A mixed T helper (Th)-1 and 2 type cytokine expression pattern was observed in all EBV positive tissue. However, in IM, PTLD and NPC a higher percentage of cells expressed IL-6 and IL-10. In all diseases except NPC cytokine production was predominantly seen in EBV positive cells. In NPC a higher percentage of infiltrating T cells expressed cytokines than the epithelial tumour cells. Overall our results show that autocrine cytokine production by EBV positive cells may contribute to the pathogenesis of EBV related diseases. However, it is plausible that the infiltrating T cells also contribute to the disease process by producing additional growth factors.

Abbreviations and Symbols

Chemical/Reagent	Abbreviation
General	
3-aminopropyltriethoxy silane	Silane
Bovine serum albumin	BSA
Carbon dioxide	CO_2
Cyclosporin A	CyA
Dextran Sulphate	DS
Diethylpyrocarbonate	DEPC
Dimethylsulphoxide	DMSO
Distilled water	dH_2O
Double distilled water	ddH_2O
Ethylene diamine-tetra-acetic acid	EDTA
Fetal calf serum	FCS
Hank's balanced salt solution	HBSS
Hydrochloric Acid	HCl
Magnesium Chloride	$MgCl_2$
Optimum tissue cutting medium	OCT
Phytohaemagglutinin	PHA
Polyvinylpyrrolidone	PVP
Proteinase K	PK
Ribonuclease	RNase
Rosewell Park Memorial Institute	RPMI
Sodium Chloride	NaCl
Sodium Hydroxide	NaOH
Solutions & Media	
Culture medium	CM
Freeze medium	FM
Neutral buffered Formalin	NBF
Phosphate buffered Saline	PBS
Phosphate EDTA	PE
Saline sodium citrate	SSC

Tris-buffered saline	TBS

Cytokines

Interferon	INF
Interleukin-2,4,6,10	IL-2,4,6,10

Cells, Cell Lines and Sera

Cytotoxic T cell	CTL
Lymphoblastoid cell line	LCL
Normal Donkey serum	NDS
Normal rabbit serum	NRS
Normal sheep serum	NSS
Peripheral blood mononuclear cells	PBMC
Reed-Sternberg cells	R/S cells
T helper cell	Th
T helper cell-1 & 2	Th-1 & 2

EBV Related Diseases

Burkitt's Lymphoma	BL
Hodgkin's Disease	HD
Infectious Mononucleosis	IM
Nasopharyngeal Carcinoma	NPC
Oral Hairy Leukoplakia	OHL
Post-transplant Lymphoproliferative Disease	PTLD
X-linked Lymphoproliferative Syndrome	X-LPS

EBV Genes

Epstein-Barr Virus Early RNAs	EBERs
Epstein-Barr Virus Nuclear Antigen	EBNA
Latent Membrane Protein	LMP

Reporter molecules, Enzymes and Enzyme Substrates

5-bromo-4-chloro-3-indolyl phosphate	BCIP
Alkaline phosphatase	AP

Diaminobenzidine	DAB
Digoxigenin	DIG
Fluorescein isothiocyanate	FITC
Horseradish peroxidase	HRP
Nitroblue Tetrazolium	NBT
Tetramethylrhodamine isothiocyanate	TRITC

Isotopes

^{51}Chromium Sulphate	^{51}Cr
[Methyl-^{3}H]-Thymidine	^{3}H-T

Units of Measurement

Becquerel	Bq
Counts per minute	cpm
Curie	Ci
G-number	g
Gram	gm
Kilogram	Kg
Litre	L
Mega (prefix)	M (prefix)
Microgram	µg
Microlitre	µl
Milligram	mg
Molarity	M
Nanogram	ng
Normal	N
Revolutions per minute	rpm

Greek Symbols

Alpha	α
Beta	β
Gamma	γ

Viruses

Epstein-Barr Virus	EBV
Herpes Simplex Type 1	HSV-1
Herpes Simplex Type 2	HSV-2
Human Cytomegalovirus	HCMV
Varicella Zoster Virus	VZV

Miscellaneous

Antibody	Ab
Antigen	Ag
Over night	O/N
Room temperature	R/T

1. Introduction

1.1 Herpesviridae

Members of the Herpesviridae exist worldwide and infect a wide range of hosts (Roizman et al, 1990). At least eight different species have been identified which infect humans. These are herpes simplex viruses (HSV) 1 and 2, Varicella-zoster virus (VZV), human cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human herpes viruses 6, 7 and 8 (HHV6, HHV7, HHV8).

1.1.1 Classification of Human Herpesviridae:

Family	Herpesviridae
Subfamily	Alphaherpesvirinae
Genus	Simplexviruses
	Herpes simplex virus type 1 (HSV1)
	Herpes simplex virus type 2 (HSV2)
Genus	Varicelloviruses
	Varicella zoster virus (HHV3, VZV)
Subfamily	Betaherpesvirinae
Genus	Cytomegaloviruses
	Human cytomegalovirus (HHV5)
Genus	Roseoloviruses
	Human herpesvirus 6 (HHV6)
	Human herpesvirus 7 (HHV7)
Subfamily	Gammaherpesvirinae
Genus	Lymphocryptoviruses
	Epstein-Barr virus (EBV, HHV-4)
	Human herpesvirus 8 (Kaposi's sarcoma associated herpesvirus, KSHV, HHV-8)

1.1.2 Physical and Biological Properties

All herpesviruses are morphologically identical. The virion consists of an icosahedral nucleocapsid, about 100 nm in diameter (Wildy et al, 1963). A lipid bilayer envelope derived from the inner nuclear membrane of the host cell,

surrounds the nucleocapsid (Morgan et al, 1959). Virus-encoded glycoproteins are incorporated into the host derived envelope. Between the capsid and the envelope is an area called the tegument, an amorphous protein layer (Morgan et al, 1968). The icosahedral capsid contains a double-stranded DNA genome in the form of a toroid (Furlong et al, 1972). The genome consists of 4 equimolar isomers with terminal and internal inverted repeats (Furlong et al, 1972). Virus enters the cell by endocytosis, the capsid is uncoated and viral DNA is released in the host cell nucleus. Viral DNA replicates in host cell nucleus and virus particles acquire an envelope by budding from the nuclear membrane (Roizman and Furlong, 1974). Virions are transported to the outside of the cell by transport through intracytoplasmic cisternae or cytoplasmic vesicles.

Primary infection with Herpesviruses is usually subclinical. Following primary infection, the virus establishes a latent infection in the host and can reactivate at any time. However, in immunocompetent individuals clinical disease after reactivation is usually localized and of short duration. Severity of symptomatic disease can increase in stressed, elderly and immunocompromised persons and may be generalized, severe, and even life threatening. Gamma-herpesviruses in particular are associated with a variety of malignancies in this scenario.

1.2 Epstein-Barr Virus

Epstein-Barr virus (EBV) or human herpes virus 4 (HHV4), is a double stranded DNA virus, which was originally visualized under an electron microscope by Sir A. Epstein in an African Burkitt's lymphoma cell line (Epstein et al., 1964). EBV is a member of genus lymphocryptovirus, subfamily gammmaherpesvirinae of the family Herpesviridae.

1.2.1 History

In 1958 Denis Burkitt first described a malignant tumour of the jaw in the malaria belt of east Africa (Burkitt, 1958). Due to the restricted geographical distribution of the tumour to certain areas of Africa, and its close association with the malaria endemic area, Burkitt suggested an infectious etiology for this tumour, transmitted by the mosquito vector. In 1964, Epstein and Barr isolated a continuous cell line from a Burkitt lymphoma tumour (Epstein & Barr, 1964) and later described the presence of a herpesvirus in these cells (Epstein et al, 1964). The discovered virus was named Epstein-Barr virus and was later recognized as the etiological agent of infectious mononucleosis (Henle & Henle, 1968; Niederman et al, 1968). EBV was also shown to immortalize lymphocytes *in vitro* (Pope et al, 1968) and cause tumours in non-human primates (Shope & Miller, 1975).

1.2.2 EBV Morphology

EBV has a DNA core inside a icosahedral nucleocapsid made up of 162 capsomere. The nucleocapsid is surrounded by the viral envelope, which has a trilaminar structure and is derived from the host cell membrane. An amorphous material termed the tegument separates the the nucleocapsid from the envelope. The most abundant EBV envelope glycoproteins are gp350 and gp220, which are encoded by the same viral gene by alternative splicing of the mRNA (Dolyniuk et al, 1976). Additional viral envelope glycoproteins expressed at much lower levels include gp85, gp25, gp42/38, gp43, and gp78/55 (Li *et al.*, 1995).

1.2.3 EBV Genome

EBV has a linear, double stranded DNA genome which is 172 kilobase pairs (kbp) in size (Dambaugh et al, 1980; Baer et al, 1984). The genome ends consist of tandemly reiterated terminal repeats (TR) of approximately 540 base pairs (bp) (Hayward et al, 1977; Given et al, 1979; Kintner et al, 1979). Within the genome several internal repeats (IR) of about 3.1 kbp join short and long unique regions (Laux *et al.*, 1985; Keiff and Leibowitz 1990; Miller 1990). The number of repeats in the DNA IR sequences varies between different EBV isolates, however this number remains constant within individual isolates (Dambaugh et al, 1980; Heller et al, 1981; Katz et al, 1988). Two types of EBV, termed EBV 1 and 2, have been identified (Bornkamm et al, 1980; Dambaugh et al, 1984) of which EBV 1 is the predominant type worldwide (Zimber et al, 1986). The two types have 70-85% homologous DNA sequences (Sample et al, 1990) with the major differences in the regions encoding nuclear antigens 2, 3, 4, and 6 and EBERS (Dambaugh et al, 1984; Rowe et al, 1989; Arrand et al, 1989). The entire EBV genome persists in the host cell as covalently closed circular episomal DNA (Lindahl et al, 1976), or very rarely as linear-integrated DNA (Fennewald *et al.*, 1984).

1.2.4 EBV Cell Tropism

Over 90% of the adult human population worldwide shows serological evidence of EBV infection (Henle & Henle, 1976). EBV has a narrow tissue tropism limited to B and T lymphocytes and epithelial cells of primate origin (Glaser et al, 1978). Recently Savard et al have also reported infection of human monocytes with EBV (Savard et al, 2000). They demonstrated presence of EBV nucleocapsids in cytoplasm by electron microscopy (EM) and EBV DNA in phagocytosis-defective monocytes (Savard et al, 2000). *In vivo* the virus enters via the nasopharyngeal epithelium and infects recirculating B lymphocytes. Earlier studies have suggested that EBV primarily infects the oropharyngeal epithelial cells and subsequently establishes a latent infection in host B cells (Sixby et al, 1984). However, It is still not clear how EBV infects epithelial cells. A recent study has reported that although cell free EBV is incapable of infecting epithelial cells, cell to cell contact between EBV infected B cells and epithelial cells in co-

culture can result in EBV infection of epithelial cell via a receptor independant pathway (Chang et al, 1999). Another recent study by Faulkner et al on a group of individuals with a rare heritable disorder, X-linked agammaglobulinemia, who lack mature B lymphocytes has shown that none of these individuals carried EBV and lacked EBV-specific memory cytotoxic T lymphocytes, suggesting that B lymphocytes, and not oropharyngeal epithelial cells, may be the target for primary EBV infection (Faulkner et al, 1999).). *In vitro* infection of B cells results in immortalization and transformation of these cells into continuously proliferating lymphoblastoid cell lines (LCL) (Pope et al, 1968). *In vitro* infection of squamous epithelial cells results in lytic viral infection with consequent virus production and cell death (Sixby et al, 1983).

1.2.5 Attachment and Entry

EBV enters B lymphocytes by attachment of viral envelope glycoprotein gp350/220 to CD21 also known as complement receptor 2 (CR2), on the B cell surface (Fingeroth, 1984; Nemerow et al, 1987). This is followed by gp85-gp25-induced fusion of the viral envelope with the cell membrane, and release of the nucleocapsid into host cell cytoplasm (Miller and Hutt-Fletcher 1992). Entry also requires the binding of another surface glycoprotein gp42 with HLA-DR (Spriggs et al, 1996) and B cells not expressing HLA-DR are resistant to infection by EBV (Li et al, 1997). Apart from binding to HLA-DR, gp42 can also facilitate viral entry into host cells by using HLA-DP and HLA-DQ as co-receptors (Haan et al, 2000).

1.2.6 Immortalization of B cells into Lymphoblastoid Cell Lines

Internalization of the virus particle by a resting B cell results in the activation of endogenous heat shock proteins (hsp)-70 and hsp-90 and P56Lck (Cheung et al, 1991; 1993). 24 hours post-infection EBV expresses nine proteins termed the latent cycle antigens. These include EBV nuclear antigens (EBNA) 1 to 6 and latent membrane proteins (LMP) 1 and 2a and 2b. Two non-translated RNAs; EBV-encoded early RNA (EBER) 1 & 2 are also expressed in early infection (Dilner et al, 1988; Roger et al, 1992). After 48 hours, the EBNA proteins reach the maximum level at which they persist throughout latent infection (Moss et al,

1981; 1986), the infected cells are enlarged and and express LMP1 induced B cell activation marker CD23 (Wang et al, 1990). The cells enter mitosis at 72 hours post infection (Sinclair et al, 1994) and continue to divide indefinitely, giving rise to a lymphoblastoid cell line (LCL), which expresses all B cell activation markers; CD23, CD30, CD39, CD70 and ki24. The type of gene expression seen in LCLs is termed a full latent viral gene expression.

1.2.7 Latent Infection

In vivo EBV infection of B cells is life long and the virus persists in the host cells in a latent form as a self-replicating extra chromosomal episome, which replicates once per cell division during S phase with transfer of one daughter molecule to each daughter cell (Yates and Guan, 1991; Mackey *et al.*, 1995). Latent infection is punctuated with periodic reactivation of viral replication and shedding of whole virions. Full latent viral gene expression is not observed in all latent EBV infections (Rowe et al, 1987). On the basis of expression of latent cycle antigens, EBV latency has been divided into three forms known as latency types I, II, and III (Rowe *et al.*, 1992; Brooks *et al.*, 1993). In type I latency EBERS 1 and 2 and EBNA1 mRNA are expressed by the virus and EBNA1 is the only latent protein synthesized. This type of latency is observed in Burkitt's lymphoma (Rowe et al, 1986). Latency II is seen in EBV-related nasopharyngeal carcinoma, T cell tumours, and Hodgkin's disease (Brooks *et al.*, 1993; Deacon et al, 1993; Minarovits *et al.*, 1994; Yoshiyama *et al.*, 1995) and is characterized by the expression of EBERS, EBNA1, LMP1 and LMP 2a and 2b. In latency III all the latent genes (EBERS 1 & 2, EBNA 1, 2, 3, 4, 5, and 6, LMP1 and LMP 2a and 2b), are expressed. Latency III is observed in EBV immortalized lymphoblastoid cell lines and EBV related lymphoproliferative disease (Brumbaugh et al, 1985).

1.2.8 EBV Encoded Latent Cycle Proteins

1.2.8.1 EBNA1

EBNA1 is a 65 to 85 kD phosphoprotein with an N-terminal and a C-terminal domain. The two domains are joined by a glycine/alanine rich internal repeat sequence of variable length. The length of the internal repeat determines the size

of EBNA1 (Kirchmaier and Sugden 1995; Mackey *et al.*, 1995). EBNA1 C-terminal domain has DNA binding properties (Ambinder et al, 1990). EBNA1 mediates viral DNA replication and is essential for maintainence of the episomal copy number during cell division (Rawlins et el, 1985; Yates *et al.*, 1985). The mechanism of activation of DNA replication by EBNA1 is not understood completely (Kirchmaier and Sugden 1995; Mackey *et al.*, 1995). EBNA1 is present in all three types of EBV latency and is expressed in all EBV related tumours (Jones *et al.*, 1989; Snudden *et al.*, 1994).

1.2.8.2 EBNA2

EBNA2 is an 86 kDa phosphoprotein, which regulates viral and cellular gene transcription by transactivation of EBV and host cell-encoded genes. EBNA2 is essential for B cell immortalisation (Cohen et al, 1989) since deletion mutants fail to immortalise B cells (Rabson et al, 1982; Hammerschmidt et al, 1989). EBNA2 transactivates EBNA1, 3, 4, 6, LMP1 and LMP 2a and 2b expression (Abbot et al., 1990; Wang et al., 1990b; Robertson et al., 1995; Cohen et al, 1989) and up-regulates expression of cellular onocogenes c-*fgr* and *bcl*-2 and B cell activation markers CD21, and CD23 (Cohen *et al.*, 1991; Wang *et al.*, 1990a; Wang *et al.*, 1991). EBNA2 does not bind DNA directly and its transcriptional transactivation function is mediated by the recombinant signal-binding protein (RBP-Jk) (Johannsen et al, 1996; Groux et al, 1997). EBNA2 binding to RBP-Jk is essential for B cell immortalisation because deletion of EBNA2 codons close to the site which interacts with RBP-Jk results in inhibition of its ability to immortalise B cells (Harada et al, 1998).

1.2.8.3 EBNA3, 4 and 6

EBNA3, 4 and 6 (also termed EBNA 3a, 3b and 3c respectively) are grouped together because they have a common origin. Like EBNA2, EBNA3, 4 and 6 interact with RBP-Jk and induce or modify transcription of genes under its control (Robertson et al, 1996; Cludts and Farrel, 1998). EBNA3, 4 and 6 compete with EBNA2 for binding with RBP-Jk and therefore have a potential to modulate EBNA2-induced transactivation of LMP1 and LMP2a and 2b. (Krauer et al, 1996;

Longnecker and Miller 1996; Bourillot et al, 1998). EBNA4 is not essential for B cell immortalisation *in vitro* (Tomkinson and Kieff, 1992) but it upregulates *bcl*2 expression and reduces the sensitivity of transformed cells to apoptosis (Silins et al, 1995). EBNA6 up regulates the expression of CD21 (Wang et al, 1990). It has also been reported that EBNA6 transfection of the Raji cell line, which has deletions in EBNA6 open reading frame, results in upregulation of LMP1 and CD23 (Allday et al, 1993).

1.2.8.4 EBNA5

EBNA5 or EBNA-LP (leader protein) is encoded from the open reading frame which forms the leader sequence of each EBNA mRNA (Wang *et al.*, 1987). It is localized in small granules within the host cell nucleus (Wang et al, 1987) where it is thought to control processing of EBV RNAs (Kieff & Liebowitz, 1990). It is suggested that EBNA5 plays a role in B lymphocyte immortalisation by stimulating and regulating EBNA2 mediated transcriptional activation (Longnecker and Miller 1996; Harada and Kieff, 1997). Recombinant viruses with deletions in EBNA5 show reduced, but still detectable, immortalising ability. EBNA5 cooperates with EBNA2 in causing transition of EBV infected cells from G0 to G1 phase during replication (Sinclair et al, 1994) and upregulates expression of LMP1 (Nitsche et al, 1997).

1.2.8.5 LMP1

LMP1 is a 58-63 kDa protein with both cytoplasmic and transmembrane domains (Leibowitz et al., 1986). LMP1 is a viral oncogene essential for B cell immortalisation (Kaye et al, 1993). LMP1 transforms rodent fibroblasts *in vitro* (Wang et al, 1985) and has been shown to induce expression of activation markers and adhesion molecules when transfected in EBV negative Burkitt's lymphoma cells or primary B lymphocytes (Wang et al, 1988; Birkenbach *et al.*, 1993). LMP1 also upregulates the expression of *bcl*-2, a cellular oncogene (Henderson *et al.*, 1991), human IL-10 (Nakagomi et al, 1994) and transcription factor NF-κB (Hammarskjold & Simurda, 1992, Huen et al, 1995) through which it exerts its effects on cytokine gene expression in host cells (discussed in detail in section

1.6). Recently it has been suggested that LMP1 contributes to the generation of Reed-Sternberg (R/S) cells in Hodgkin's disease by down-regulating the expression of CD99. In LCLs this leads to generation of cells with morphology and functions identical to those of R/S cells (Kim et al, 2000).

1.2.8.6 LMP 2a and 2b

LMP2a and 2b are 53 and 40 kDa proteins encoded by two mRNAs that have eight common exons (Longnecker *et al.*, 1991). LMP2a is thought to prevent lytic viral replication in latently infected B cells (Miller et al, 1994). LMP2b is thought to downregulate this activity of LMP2a. LMP2a and 2b are under the control of separate promoters and differential expression may result in either lytic activation or maintenance of latent infection (Longnecker and Miller 1996). Although previously it has been reported that LMP2a and 2b are essential for efficient transformation of B cells (Brielmeier et al, 1996), recently Speck et al, have reported that recombinant EB virus lacking LMP2a and 2b immortalises B cells as efficiently as the wild type virus and thus shown that LMP2a and 2b are not involved in B cell transformation by EBV (Speck et al, 1999).

1.2.8.7 EBER1 and EBER2

EBV encodes two non-translated Epstein-Barr virus-associated early RNAs known as EBER1 and 2. Both EBERs are transcribed by RNA polymerase III and are the most abundantly expressed RNAs in all three forms of EBV latency (approximately 10^7 copies per cell). It is suggested that EBERs are involved in the transcription and processing of viral RNA and are associated with initiation of transcription of all other viral mRNAs (Glickman *et al.*, 1988). Previously it has been reported that EBERs are not required for *in vitro* B cell immortalisation and virus replication (Swaminathan et al, 1991). However, it has recently been shown that EBERs have oncogenic functions in BL cells and are responsible for the malignant phenotype and resistance to apoptosis in the BL cell line Akata (Komano et al, 1999). EBER gene transfection into EBV-negative Akata lines restored their capacity to grow in soft agar, tumorigenicity in SCID mice, resistance to apoptotic inducers, and upregulated expression of bcl-2 oncoprotein.

1.2.9 Lytic Cycle

EBV infection of B-lymphocytes is predominantly latent. Infection of epithelial cells can be latent, as in NPC, but predominantly leads to complete viral replication resulting in cytolysis (Sista *et al.*, 1995). In the lytic cycle (virus-productive), the synthesis and expression of EBNA 1-6, LMP and LMP 2a and 2b is followed by expression of EBV lytic cycle genes in a sequential manner. Lytic cycle genes are classified as immediate early, early and late genes. Each set activates the expression of the next set. There are two immediate early genes: BZLF1 and BRLF1. Early genes include the early antigens (EA), which are of two types: EA-diffuse and EA-restricted (EA-D & EA-R). The EA-R is only found in the nucleus and EA-D is found both in the nucleus and the cytoplasm (reviewed in Crawford et al., 1987). Expression of early genes is followed by viral DNA synthesis and finally the late genes are expressed which are structural proteins.

1.3 Primary EBV Infection

Primary infection is usually asymptomatic in children although it might be accompanied by mild pharyngeal disease (Henle et al, 1979). However, if primary infection is delayed till early adulthood (15-25 years) it is often symptomatic and manifests as infectious mononucleosis or glandular fever (Henle & Henle, 1979).

1.3.1 Infectious Mononucleosis (IM)

EBV was first identified as the cause of IM in 1968 (Henle et al, 1968; Niederman et al, 1968).

1.3.1.2 Pathogenesis

IM is a disease mainly of the adolescents and EBV transmission occurs via salivary exchange during kissing. The B cells in the lymphoid tissue of the pharynx get infected and from this site the EBV infected B cells are disseminated throughout the body via the blood stream. Primary EBV infection results in a strong but self-limited, immune response in the host, associated with appearance of a variety of immunoglobulins (including the heterophile antibody, auto-antibodies and antibodies to EBV antigens) and expansion of EBV-specific CTLs

in the host. The exaggerated immune response (mainly CD8 T cells) is thought to contribute to the clinical symptoms of IM (Foss et al, 1994).

1.3.1.3 Clinical Manifestations

The incubation period of IM is estimated to be 30-50 days. Classic symptoms include sore throat, fever and fatigue. Common clinical signs observed include lymphadenopathy, splenomegaly, hepatomegaly, eyelid edema, exanthems, and enanthems. Signs and symptoms can be abrupt or may be preceded by a prolonged prodromal period of fever with mild pharyngitits. Lymph node involvement is bilateral and most commonly involves the posterior and anterior cervical chains. Less commonly the axillary and inguinal nodes may be involved (Schooley, 1955). IM is usually self limiting and resolves in 1-2 weeks, but in 1:2000 cases it can become chronic (Virelizier et al, 1978). Protracted or recurrent symptoms of infectious mononucleosis are called chronic active mononucleosis and this condition is characterised by fatigue, fever, pharyngitis, hepatitis and depression (Andersson, 1991). The syndrome may continue for months and years following typical IM with persistent serological evidence of primary EBV infection (Okano et al., 1988).

Acute IM patients show moderate peripheral leukocytosis (>20,000/mm^3). More than 50% (or greater than 4,500/mm^3) of these leukocytes are lymphocytes (mainly CD8 positive T cells) of which at least 10% (or greater than 1,000/mm^3) are atypical lymphocytes previously referred to as Downy cells. The lymphocytosis is due to EBV infection of B-cells followed by anti-EBV T and NK cell proliferation. Mild thrombocytopenia occurs in around 50% cases with platelet counts ranging from 100,000 to 140,000/ mm^3. 90% cases may develop a mild hepatitis in the second or third week with up to two-fold increase in hepatic enzymes: lactic dehydrogenases, alkaline phosphatase and the transaminases.

1.3.1.4 Complications

Complications are rare after acute IM but can be serious. These include upper airway obstruction (Wolfe et al, 1980), rash associated with ampicillin administration (Patel, 1968, Pullen et al, 1967), splenic rupture and neurologic

complications such as Guillain-Barré syndrome (Reller, 1973), cranial nerve palsies, aseptic meningitis and meningoencephalitis (Grose et al, 1975, Domachowske et al, 1996, Eshel et al, 1987).

1.3.1.5 Diagnosis

IM is routinely diagnosed by serological detection of the heterophile antibody (McDougal, 1978). Heterophile antibody is of IgM class and is present in 60-70% of patients in the first week of clinical illness and can persist for up to 12 months. IgG and IgM antibodies directed against EBV-specific antigens can be detected by immunofluorescence assays, enzyme immunoassays, and immunoblot techniques. Presence of IgM anti-VCA and absence of antibodies to EBNA is diagnostic of acute, primary infection.

1.4 Immune Response to EBV

The first line of defence against the virus is interferon, secreted by virally infected B cells. It may control EBV-induced B cell proliferation by antiviral replication activity and enhancement of natural killer (NK) cell activity. (Lotz et al., 1986).

1.4.1 Humoral Immune Response to EBV

The first antibodies to appear after EBV infection are the anti-VCA IgM and IgG. These antibodies appear before the onset of clinical disease and the IgG response persists for life (reviewed in Crawford et al., 1987). Anti-EA-D IgG antibodies develop transiently and disappear during convalescence. However, in some cases they may persist at low titres (Linde, 1992). EA-R antibodies are rarely present in acute infection (de Thé, 1982). Anti-EBNA1 IgG are not present in acute infection and are only detected weeks to months post infection and then persist indefinitely (Henle et al., 1987).

1.4.2 Cellular Immune Response to EBV

The anti-EBV cellular immune response involves both NK cells and EBV antigen-specific T cells which have the potential to kill EBV infected cells (Callan et al, 1998). NK cell killing of EBV infected cells is both direct and antibody dependent, however, a higher effector to target ratio is required for target lysis as

compared to CTLs (Masucci et al, 1981). EBV-specific CD8 positive CTLs recognize HLA class I associated EBV antigen epitopes presented on B cells or other antigen presenting cells (APC). These persist in the immunocompetent host as CTL precursors (CTLp) with frequencies ranging between 1/400-1/42,000 of the total T cell population (Kusunoki et al, 1993). HLA class II restricted anti-EBV CD4-positive T cells also appear during primary EBV infection but are present at much lower levels as compared to the CD8 positive CTLs (Misko et al 1984). The CTL response to different EBV latent antigens is not equal and in majority of cases CTLs are primarily directed against immunodominant epitopes within primary sequences of EBNA 3, 4 and 6 (Khanna et al, 1992, Murray et al, 1992, Rickinson, 1998). A lesser degree of response is seen to EBNA 2, 5, LMP1 and LMP 2a and 2b (Gavioli et al 1992, Khanna et al, 1992, Murray et al, 1992, Khanna et al, 1998). Dominance of response against particular antigen epitopes depends upon level of expression of the epitope on the host cell surface. Lee et al studied the level of expression of HLA-A-11 restricted EBNA4 epitopes and found that the dominant HLA-A-11 epitope was presented on the cell surface at a frequency higher than that of other HLA-A-11 restricted EBNA4 epitopes (Lee et al, 1995). Immune dominance of an epitope is also influenced by the efficacy with which it binds to HLA molecules (Khanna et al, 1997). Non-binding to HLA molecules is thought to be the reason why CTL response to EBNA1 is rare and has only been detected in occasional cases (Blake et al, 1997). EBNA1 contains a 200 residue long glycine-alanine repeat region, which prevents its degradation by the host cell and prevents complexing of EBNA1 antigenic epitopes with HLA molecules for presentation at the host cell surface (Levitskaya et al, 1997; Sharipo et al, 1998). Various HLA class I restricted immunodominant epitopes have been identified in EBV antigens (Murray et al, 1992). A list of immunogenic EBV antigen epitopes, their position in the primary antigen sequence and HLA restriction is shown in table 1.1. A strong CTL response to EBV lytic antigens is also seen during primary infection. This response is stronger than that to latent cycle antigens during the primary infection but the numbers of lytic antigen-specific CTLs fall after acute infection (Steven et al, 1997, Callan et al, 1998, Tan et al, 1999, Khanna et al, 1999). However, CTLs directed against immediate early genes: ZEBRA and BHRF-1 are maintained at significant frequencies after acute

infection has subsided (White et al, 1996). This may serve to limit the number of B cells undergoing lytic viral infection and thus control virus production.

Table 1.1

Defined T cell immunogenic epitopes of EBV-encoded proteins and presenting HLA-alleles

EBV Antigen	HLA Allele	Residue	Epitope Sequence
LMP-2	A0201	426-434	CLGGLLTMV
	A0201	329-337	LLWTLVVLL
	A0206	453-461	LTAGFLIFL
	A1101	340-349	SSCSSCPLSK
	A2402	419-427	TYGPVFMCL
	B2704	236-244	RRRWRRLTV
	B60	200-208	IEDPPFNSL
EBNA-6	B4405 >03 >02	281-290	EENLLDVFRM
	B37	285-293	LDFVRFMGV
	A2	284-293	LLDFVRFMGV
	B62	213-222	QNGALAINTF
	B7	881-889	QPRAPIRPI
EBNA-4	A11	399-408	AVFDRKSVAK
	A11	416-424	IVTDFSVIK
EBNA-3	B35	458-466	YPLHEQHGM
	B8	325-333	FLRGRAYGI
	B8	158-166	QAKWRLQTL
	A2	596-604	SVRDRLARL
	A3	603-611	RLRAEAGVK
	B7	379-387	RPPIFIRRL
	A24	246-253	RYSIFFDY
BZLF1	B8		RAKFKQLLQ
BMLF1	A2		GLCTLVAML

(Adapted from O'Reilly et al, 1998)

1.5 EBV Related Malignancies

1.5.1 Post-transplant Lymphoproliferative Disease (PTLD)

Post-transplant B cell lymphoproliferative disorders (PTLD) include a variety of lymphoid hyperplasias and neoplasms that are observed in organ transplant recipients. These opportunistic neoplasms occur as a consequence of the immunosuppression, which is required to prevent graft rejection (Brumbaugh et al, 1985, Swinnen, et al 1995). EBV is thought to be an important cofactor in the development of PTLD and is present within the malignant B cells in 90% cases (Chadburn et al, 1997). EBV related PTLD occurs in approximately 1-2% bone marrow, 1% renal, 2% liver, 5-9%, heart/lung, 2-10% heart and 19% of intestinal transplant recipients (Chen et al, 1993; Reyes et al, 1996). PTLD tumour cells are phenotypically similar to LCL cells in that they express all EBV latent antigens and B cell activation marker CD23 (Young et al, 1989; Thomas et al, 1990).

PTLD most commonly arises within the first post-transplant year (Penn, 1994). The tumour may arise in lymphoid tissues such as tonsils or lymph nodes, but often occurs in extranodal sites. PTLD can arise from either donor or recipient cells but most commonly arises from recipient cells in solid organ transplant patients and donor cells in bone marrow recipients (Chadburn et al, 1995; Weissmann et al, 1995; Zutter et al, 1988). Involvement of the transplanted organ in the malignant process is reported in 30-40% of cases (Cohen, 1991).

1.5.1.1 Histology and Classification of PTLD

Histologically PTLD can range from hyperplastic lesions or atypical lymphoid lesions, to aggressive B cell lymphomas, which are usually of the non-Hodgkins variety (Harris et al 1997). PTLD was first classified into four separate histological categories in 1981 by Frizzera et al (Frizzera et al, 1981): polymorphic diffuse B cell hyperplasia, polymorphic B cell lymphoma, reactive lymphoid hyperplasia and immunoblastic sarcoma. In 1988, Nalesnik separated monomorphic tumours from the polymorphic form of the disease (Nalesnik et al, 1988) and in 1995, Knowles et al combined tumour histology with molecular genetic analysis and classified PTLD into: reactive hyperplasia of plasma cells,

monoclonal polymorphic hyperplasia and polymorphic lymphoma and true monoclonal lymphomas with possible alterations in proto-oncogenes and/or tumor suppressor genes (TSG) (Knowles et al, 1995). In 1997 Harris et al added plasmacytomas, T cell rich B-cell lymphomas, and T cell lymphomas to the PTLD classification (Harris et al, 1997).

1.5.1.2 Pathogenesis of PTLD

The association of PTLD tumours with EBV was first shown by detection of EBNA antigen in tumour sections (Crawford et al, 1980) and later by the demonstration of EBV DNA within PTLD tissue (Weiss & Movahed, 1989). Immunosuppressive drugs used in transplantation have been shown to suppress EBV-specific CTL response *in vitro* (Crawford et al, 1981; Tsoukas et al, 1982; Burman & Crawford, 1991) and very low EBV-specific CTL activity has been reported in transplant recipients during immunosuppression (Haque et al, 1997). In 1-10% cases this results in an uncontrolled proliferation of EBV transformed B-cells, causing PTLD (Penn, 1989). Graft recipients who are EBV negative prior to transplantation but seroconvert after the procedure are at particular risk because they do not have a memory CTL response to EBV.

EBV latent gene expression in most PTLD tumours is latency type III (Thomas et al, 1990; Young, 1989). However, latency types II and I have been observed in some cases (Thomas et al, 1990; Gratama et al, 1991; Delecluse et al, 1995). Although most transplant recipients are EBV seropositive, the occurrence of PTLD in only a small proportion of these cases is suggestive of additional factors required for PTLD development. The tumour frequently occurs in the GI tract and the transplanted organ, which are both sites of chronic antigenic stimulation and are rich in cytokines (Chen et al, 1993; Cohen, 1991). Tumours are infiltrated with CD4+ T cells, which might provide growth factors for tumour development (Perera et al, 1998). Serum levels of Th type 2 cytokines: IL-4, 6 and 10 have been reported to be elevated in PTLD patients as compared to normal individuals (Tosato et al, 1993; Mathur et al, 1994) and IL-4 and 10 mRNA has been detected in PTLD tissue by reverse transcriptase-polymerase chain reaction (RT-PCR)

(Nalesnik et al, 1999). These cytokines are B cell growth factors and might contribute to the pathogenesis of the disease.

1.5.1.3 Treatment of PTLD

1.5.1.3.1 Conventional Treatment

Reduction or discontinuation of immunosuppression is the standard first line treatment for PTLD (Starzl et al, 1984, Kawa et al, 2000). This allows the host immune system to recover and mount an anti-EBV response. In a study by Malatack et al less severe disease such as lymphadenopathy was controlled by 50% reduction in immunosuppression but lymphomas and disseminated disease required complete withdrawal of immunosuppressive drugs (Malatack et al, 1991). However, withdrawal of immunosuppressive drugs can result in allograft rejection (Swinnen, 1995). This can be avoided by giving the minumum dose of immunosuppressive therapy required for prevention of graft rejection (Swinnen, 1995). Anti-virals such acyclovir and gangcyclovir have been used successfully to control PTLD in certain studies (Darenkov et al, 1997). However both these drugs have no effect on latent virus infection and their role in treatment of PTLD is dubious. Alpha-interferon has also been used effectively to induce regression in PTLD tumours in transplant recipients (Davis et al, 1998; Faro et al, 1996). Multi-agent chemotherapy induced remission of PTLD in 75% cases in a study by Swinnen et al on cardiac transplant recipients (Swinnen et al, 1997). In another study Balfour et al reported remission of PTLD in solid organ transplant patients with a combination of cyclophosphamide and prednisone. (Balfour et al, 1999). Recently, Schoch et al reported successful treatment of a lung transplant recipient undergoing acute graft rejection and suffering from EBV-positive PTLD, with a combination of extracorporeal photochemotherapy and moderate reduction of immunosuppressive therapy (Schoch et al, 1999).

1.5.1.3.2 Adoptive Cellular Immunotherapy

Cellular immunotherapy was introduced in 1992, when human cytomegalovirus (HCMV)-specific CD8+ T cells were grown *in vitro* and used to reconstitute immunity to human cytomegalovirus (Riddell et al, 1992). Since then major

advances have been made in the field of adoptive cellular therapy for many human diseases particularly cancers including malignant melanoma, ovarian carcinoma (Han et al, 1999, Hayashi et al, 1999), colorectal cancer (Miyazono et al, 1999), bladder cancer (Peralta et al, 1999), HPV related cervical carcinoma (Schoell et al, 1999), EBV-related Hodgkin's disease (Roskrow et al, 1998) and PTLD (Boyle et al, 1993; Papadopoulos et al, 1994; Rooney et al, 1995; Khanna et al, 1999). Several studies have demonstrated that PBMC or *in vitro* expanded EBV specific T cells from transplant donors can control growth of PTLD tumours (Heslop et al, 1994; Heslop et al, 1996). In 1994 Papadopoulos et al reported complete regression of EBV related PTLD in five bone marrow transplant recipients with repeated small dose infusion of donor PBMC. However, graft-versus-host disease (GvHD) was a complication observed in all cases in this study due to the alloreactive T cells in unfractionated donor PBMC (Papadopoulos et al, 1994). In 1997 Nalesnik et al acheived regression of PTLD in four transplant patients who were unresponsive to reduction in immunosuppression by using PBMC cultured *in vitro* with IL2 for 10-12 days (Nalesnik et al, 1997). Phenotypic analysis showed that the expanded cells had large populations of both T and NK cells, which were specific for both autologous and allogeneic EBV transformed cells and demonstrated a strong cytotoxicty to K562 cells, a leukaemic cell line sensitive to NK cell killing (Nalesnik et al, 1997). To reduce the problem of GvHD in PTLD patients, the use of *in vitro* grown virus-specific T cells, which have been stimulated with autologous cells presenting viral target antigens was first reported by Rooney et al in 1995. They used *in vitro* generated donor EBV-specific T cells for prevention of PTLD in pediatric bone marrow transplant (BMT) recipients. T cell lines were grown and expanded *in vitro* with autologous (donor) irradiated LCL cells as stimulators. T cells were expanded for 21-28 days to select the EBV-specific T cell clones and potentially reduce the population of alloreactive T cells. To trace the fate of the infused T cells *in vivo*, the cells were labeled with a retroviral vector carrying a neomycin resistance gene (neo^R) . Three BMT patients with PTLD of donor cell origin were treated with the labeled T cells. All the three patients showed regression of disease (Rooney et al, 1995). In another study the same group prophylactically infused 25 BMT recipients with neo^R-marked donor CTLs. None of the patients developed PTLD in contrast to the

historical non-CTL treated transplant group in which 19% (5 out of 27) developed PTLD. No GvHD was observed in patients infused with the EBV-specific CTLs and the cells could be detected in the peripheral blood of recipients for a periods of 18 months (Heslop et al, 1996).

All these studies were carried out in BMT recipients where the tumour is of donor cell origin and the donor is healthy and available for providing CTLs. However, PTLD tumours in solid organ transplant recipients originate from recipient cells (Cen et al, 1991; Weismann et al, 1995) and the donor is often cadaveric or not readily accessible. Therefore, logical approaches to cellular immunotherapy of PTLD in this scenario would be to either store recipient PBMC prior to transplantation and immunosuppression or to use *in vitro* expanded EBV-specific allogeneic HLA-matched CTLs (reviewed in Haque et al, 1999). The feasability and safety of *in vitro* generated autologous EBV-specific CTLs for control of PTLD has been studied by Haque et al in three healthy solid organ transplant recipients (Haque et al, 1998). Infusions of escalating doses of CTLs were delivered at fixed intervals to each patient post transplant. EBV-specific CTL precursor (CTLp) numbers rose higher than those measured in pre-transplant samples and remained high till 3 months after the last infusion. CTLp were functional and EBV-specific when tested in standard chromium release assays. Compared to pretransplant levels, EBV DNA levels rose post transplant but dropped to levels lower than pretransplant levels after CTL infusions and remained low for 3 months post infusion. The patients remained healthy and no GvHD was observed (Haque et al, 1998). In another study autologous EBV-specific CTLs generated from an EBV seronegative solid organ transplant recipient, who seroconverted post transplant and developed PTLD, have been successfully used to cause regression of the tumour (Khanna et al, 1999).

Due to the non-likelihood of obtaining pre-transplant PBMC samples from all transplant patients interest is now turning more towards PTLD therapy with *in vitro* grown HLA-matched but allogeneic CTLs (reviewed in Haque et al, 1999) which could serve as an inexpensive and a less labour intensive alternative to growing autologous CTLs from all transplant patients. The same group is now in the

process of developing an EBV-specific CTL bank from EBV-seropositive healthy donors of different HLA types. This would provide immediate cellular therapy for PTLD cases refractory to conventional treatment (Haque et al, 1999).

In contrast to PTLD, which expresses the full range of latent viral proteins, other EBV-related malignancies (BL, NPC, HD) do not express all the EBV genes (Kerr et al, 1992; Ambinder & Mann, 1994; Rickinson et al, 1998). CTL responses to EBNA1 have not been observed because of lack of presentation of EBNA1 immunogenic epitopes on host cell surface (Kerr et al, 1992, Wrightham et al, 1995). However, studies have shown that the malignant epithelial cells in NPC and Reed-Sternberg cells in EBV positive HD express LMP1 and 2 along with EBNA1 (Khanna et al, 1998; Sing et al, 1997). Cells expressing immunodominant epitopes from within the primary LMP1 and 2 sequences could serve as potential targets for CTLs specific for these epitopes. Although CTL response to LMPs constitiutes a minor proportion of overall EBV-specific CTL response, recently CTL target epitopes have been identified in LMP1 (Khanna et al, 1998) and LMP2a and 2b (Lee et al, 1997, Khanna et al, 1999). *In vitro* expanded CTLs targeted to these epitopes have been shown to effectively lyse cells expressing these epitopes (Khanna et al, 1999). Therefore, selective *in vitro* expansion of epitope-specific CTLs could form the basis of a CTL therapy for EBV related malignancies which have a restricted pattern of EBV gene expression.

1.5.2 EBV related Nasopharyngeal Carcinoma (NPC)

NPC is a rare squamous cell carcinoma of the nasopharynx (Old et al, 1966) with a world wide annual incidence 1 per 100,000 persons (Hirayama, 1978). However, compared to the rest of the world it has a higher incidence in China, Africa, Alaska and Greenland (Muir et al, 1972) with the highest incidence in Southern Chinese of 23.3 and 8.9 per 100,000 for males and females respectively (Simons et al, 1982; Muir et al, 1972; Fandi et al 1994).

1.5.2.1 Histological Appearance and Classification

Electron microscopy has shown that all NPC tumours are of squamous epithelial cell origin (Ensley et al, 1986, Fandi et al, 1994). However, tumours differ in their histological appearance and are classified into two main histologic types. 70% tumours are undifferentiated non-keratinising type, 30% show squamous differentiation (Niedobitek et al, 1991).

1.5.2.2 Aetiology & Pathogenesis

NPC has a multifactorial aetiology. It shows a strong association with EBV (Neel et al, 1984). EBV genome and expression of EBV latent antigens have been found in both non-differentiated and in well differentiated NPC tumours (Anderson-Anvert et al, 1979; Raab-Traub et al, 1987). EBV in NPC tumours shows type II latency with expression of EBERS, EBNA1, LMP1 and LMP2a and 2b (Fahraeus et al, 1988; Brooks et al, 1992). Environmental factors such as tumour-promoting factors in regional food are also thought to predispose to NPC. Herbal remedies made from plant extracts of the families *Euphorbiaceae* and *Thymeliaceae* containing phorbol esters, and childhood consumption of salted fish containing carcinogenic volatile nitrosamines, mainly N-nitrosodimethylamine and N-nitrosodiethylamine, have been suggested as a risk factors (Hirayama, 1978; Yu, 1990). It is suggested that both these environmental factors interact with EBV by increasing its replication and facilitating the oncogenic effect of LMP1. More recently Ho et al have reported NPC occurence in three workers in Taiwan who faced prolonged exposure to sulphuric acid vapour (Ho et al, 1999). NPC also has a higher incidence in individuals with the HLA types A2, Aw33, Bw46, B58, DR9 Cw11, C3 (Chan, 1990) and DR3 (Mellins et al, 1990). HLA A2, Cw11, Bw46 with a missing DR9 locus are associated with old age onset (over 30 years of age) of NPC and indicate a better prognosis than the early age onset variety (below age of 30 years), which is associated with HLA Aw33, C3, Bw58 and DR3 (Chan et al, 1983; 1990; Zhu et al, 1990).

1.5.2.3 Signs and Symptoms

Most commonly NPC presents as unilateral or bilateral, painless, enlarged cervical lymph nodes (approximately 75% of patients) (Sham et al, 1990). Upper

cervical nodes are the first to be affected and nodal involvement progresses in a cephalad-caudal direction (Sham et al, 1990). Other common symptoms are nasal obstruction, epistaxis, diminished hearing (Su & Juan, 1985), tinnitus and recurrent otitis media (Chowdhury et al, 1988). Neurological involvement indicates advanced local disease and commonly causes headache and cranial nerve dysfunction (usually V-VI or IX-XII), which can present as altered facial sensations, diplopia, trigeminal neuralgia and Horner's syndrome (Skinner et al, 1991).

1.5.2.4 Diagnosis and Treatment

NPC is diagnosed by visual examination (mirror or endoscopic) and biopsy of the nasopharyngeal mass, computed tomographic (CT) scan or magnetic resonance imaging (MRI) (Vogl et al, 1990). Serological detection of anti-VCA IgA supports the diagnosis of EBV related NPC (Tam et al, 1990). Smaller tumours can be cured by radiation therapy, with survival rates of 80% to 90% (Bailet et al, 1992). Large or disseminated tumours or tumours not responding to radiotherapy, are treated with concurrent chemotherapy with cisplatin and 5 flourouracil (Ong et al, 1999; Chua et al, 2000). Prognosis depends upon the size of the tumor, stage, and involvement of neck nodes (Sanguineti et al, 1997). Advanced disease, associated with cervical lymph node, cranial nerve, and bone involvement are often difficult to control and distant metastases occur despite local control (Fandi et al, 1994; Fandi et al, 2000; Teo et al, 1999).

1.5.3 Hodgkin's Disease (HD)

Hodgkin's disease is a lymphoid malignancy first described by Thomas Hodgkin (Hodgkin T, 1832). Histologically HD is distinct from other lymphomas because of the presence of multinucleated giant Reed-Sternberg (R/S) cells (Reed, 1902). R/S cells are characteristic of HD and contain two or more nuclei of large size with large inclusion-like nucleoli. Nucleoli are surrounded by a clear zone with homogeneous eosinophilic or amphophilic staining characteristics. Due to rarity of R/S cells in HD comprehensive analysis of their origin has not been possible. However, recently Cossman et al reported similarities between gene expression of R/S cells and germinal center B cells and have suggested that R/S cells are

derived from a B cell lineage (Cossman et al, 1999). The R/S cells can vary in their morphological appearance, depending on the histological subtype.

1.5.3.1 Cellular Classification

Pathologists currently use the Rye modification of the Lukes' classification for the histologic classification for adult Hodgkin's disease (Lukes et al, 1966)

Classic HD

Nodular sclerosis, Mixed cellularity and Lymphocyte depletion

Non-Classic HD

Lymphocyte predominant

1.5.3.1.1 Nodular sclerosis

In the nodular sclerosis type HD thick fibrous bands divide the lymph node into cellular nodules containing the neoplastic proliferation. The external fibrous capsule is also thickened. Lacunar cells are a variety of R/S cells and a feature unique to nodular sclerosis HD.

1.5.3.1.2 Mixed Cellularity

Mixed cellularity HD has a background of inflammatory cells comprised mainly of lymphocytes, eosinophils, neutrophils, histiocytes and plasma cells. Compared to other HD types this has more numerous R/S cells, and has a slightly worse prognosis.

1.5.3.1.3 Lymphocyte Depletion

In the lymphocyte depletion HD necrosis is prominent in the germinal centers, surrounded by R/S cells. Some cases of lymphocyte depletion contain infiltrating atypical cells, sometimes referred to as sarcomatoid cells.

1.5.3.1.4 Lymphocyte Predominant

Lymphocyte-predominant HD is distinct from classic disease. R/S cells are very rare and frequently absent and the immunophenotype is CD15-, CD20+, CD30-,

CD45+, while the profile for classic HD is CD15+, CD20-, CD30+, CD45- (von Wasielewski et al, 1997; Bodis et al, 1997). Although the classical and lymphocyte predominant varieties of HD differ in their immunophenotype, no significant difference is observed between their clinical response or outcome to standard therapies (Diehl et al, 1999).

1.5.3.2 Aetiology of HD

60% of HD cases are associated with EBV. However, the exact relationship between EBV and pathogenesis of HD is still unclear. The first evidence of EBV involvement in the pathogenesis of HD came in the early seventies when different scientific groups detected high titre anti-EBV antibodies in sera of patients suffering with HD (Goldman et al, 1970; Johansson et al, 1970). EBV genome and latent EBV genes have been detected in both R/S and mononuclear HD cells (Karameris et al, 1992) and EBV genome was found in both mixed cellularity and nodular sclerosing varieties of HD (Boiocchi et al, 1992). The risk of developing HD 5 years post acute IM infection is increased 5 to 6 folds as compared to controls (Mueller et al, 1989).

1.5.3.3 Clinical Manifestations and Disease Staging

The most common presentation of HD is painless lymphadenopathy, usually involving the cervical nodes but occasionally the axillary or inguinal/femoral nodes. Constitutional symptoms such as irregular low grade fever, drenching night sweats, and over 10% weight loss can preceed clinical HD. Thoracic disease may present with cough, chest pain, dyspnea, and, rarely hemoptysis. Bone involvement can present as bone pain and/or back pain and signs and symptoms of spinal cord compression. Anemia and splenomegaly with hypersplenism may also be seen.

The staging classification currently in use for HD was adopted in 1971 at the Ann Arbor Conference (Carbone et al, 1971) and later modified at the Cotswolds meeting (Lister et al, 1989). HD is divided into four stages based on a combination of clinical and pathologic features of the disease. Stages are further subclassified into A and B categories: Category A includes patients with no

clinical symptoms and category B includes cases with defined general symptoms: unexplained loss of more than 10% of body weight in the 6 months before diagnosis, unexplained fever with temperatures above 38 degrees Celsius, drenching night sweats (Carbone et al, 1971)

Stage I

Stage I involves a single lymph node region or localized involvement of a single extralymphatic organ or site.

Stage II

Stage II involves 2 or more lymph node regions or a single associated extralymphatic organ or site and its regional lymph node(s) with or without involvement of other lymph node regions on the same side of the diaphragm.

Stage III

Stage III involves lymph node regions on both sides of the diaphragm with or without involvement of an associated extralymphatic organ. Splenic involvement is included in stage III disease.

Stage IV

Stage IV involves disseminated (multifocal) disease on one or both sides of diaphragm accompanied by distant (nonregional) nodal involvement.

1.5.3.4 Treatment and Prognosis

Without treatment HD has a 5 year survival rate of 5% however, chemotherapy and radiotherapy achieve cures in up to 70% of cases. In early disease radiation therapy is delivered locally to clinically involved sites and to regions of initial nodal involvement (Sears et al, 1997). Paediatric or advanced-staged adult patients also receive chemotherapy in conjunction with radiotherapy. EBV-specific cytotoxic T cell therapy has also been used to treat relapsed EBV positive HD. However, the success rate has not been as good as that for PTLD. Rooney et al, generated autologous EBV specific CTLs from 11 HD patients and treated 3 with gene-marked EBV-specific CTLs. All three patients showed transient recovery from clinical symptoms but relapsed during or after termination of CTL treatment (Rooney et al, 1998).

1.5.4 Burkitt's Lymphoma (BL)

Burkitt's lymphoma is one of the fastest growing human cancers. It is a monoclonal B cell neoplasm characterized by small non-cleaved cells that express surface IgM (Pelicci et al, 1986). BL cells contain one of three cytogenetic abnormalities involving a translocation between chromosomes 8 and 14, chromosomes 8 and 22 or chromosomes 2 and 8. The abnormality is a balanced, reciprocal translocation of genetic material from the long arm of chromosome 8 to the long arm of chromosome 2, 14 or 22 and results in dysregulation of c-myc oncogene on chromosome 8 by bringing it under the control of an immunoglobulin gene promorter on chromosomes 14, 22 or 2 (Bernheim et al, 1981). BL has two variants: African and non-African (also referred to as endemic and sporadic). Both types are histologically and cytogenetically similar but have different epidemiologic patterns and clinical presentations.

1.5.4.1 Pathogenesis

EBV is associated with the African type with EBV genome detected in 97% cases (zur Hausen & Shulte-Holthausen, 1970; Anderson et al, 1976). BL cells display type 1 EBV latency; EBERS 1 and 2 and EBNA1 are the only genes expressed (Rowe et al, 1986). Another co-factor associated with African BL pathogenesis is plasmodium falciparum malaria (Burkitt, 1969). It is suggested that hyperendemic-malaria induced immunosuppression contributes to EBV-driven B cell proliferation in BL patients. High turnover of B cells in the bone marrow is thought to result in one of the three chromosomal translocations specific to BL (Lenoir & Bornkamm, 1987).

1.5.4.2 Clinical Features and Treatment

African BL is more common in male than female children with the mean age of onset of 7 years (Quinn, 1983). It can occur at any site in the body but 70% present as a rapidly growing jaw or orbital mass. Less commonly BL can present as an abdominal mass involving the kidneys and ovaries, however, nodal and bone marrow involvement is not usual (Burkitt, 1958, Burkitt & O'Conner, 1961). The tumour is rapidly growing, firm and painless and if not treated is invariably fatal (Burkitt, 1983). The alkylating agent cyclophosphamide is the drug of choice for

the treatment of BL (Ziegler et al, 1970) which induces complete sustained remission in 50% cases, especially if tumours are small.

1.5.5 X-linked lymphoproliferative Syndrome (X-LPS) or Duncan's disease

This is a rare familial disorder, which occurs in people carrying a defective gene on chromosome X, which selectively disables the host's immune control of EBV infection resulting in uncontrolled proliferation of EBV-infected lymphocytes (Purtilo et al, 1974, Skare et al, 1987). This causes fatal IM with liver disease in 70% of those affected (Jager et al, 1998). Some patients survive the initial infection but later develop fulminant lymphoproliferative disease (plasmacytoma, B cell and histiocytic lymphoma), hypogammaglobulinemia and other immunologic abnormalities (Purtilo et al, 1982, Mroczek et al 1987). The genetic abnormality associated with X-LPS has been localized to the Xq25 (Maia & Garawacki, 1999). A recent study by Sayos et al suggested that dysregulation of T/B-cell interactions induced by signaling lymphocyte activation molecule (SLAM), due to the absence of its inhibitor SLAM-associated protein (SAP), leads to an inability to control EBV driven B-cell proliferation (Sayos et al, 1998). SLAM is a glycoprotein belonging to the immunoglobulin gene superfamily (Cocks et al, 1995). It is expressed by both B and T cells and is a potent stimulator of cell activation and proliferation (Punnonen et al, 1997). The gene for SAP has been mapped to the same area as the X-LPS gene and mutations in SAP have been identified in patients with X-LPS (Sayos et al, 1998). Unless treated X-LPS inevitably results in death by age of 40 (Grierson & Purtilo, 1987).

1.5.6 Oral hairy leukoplakia (OHL)

OHL is an EBV associated epithelial lesion associated with immunodeficiency and most commonly occurs in AIDS patients (Wurapa et al, 1999) but is also observed in transplant recipients (Greenspan et al, 1985). It is found predominantly on the lateral margins of the tongue, but can also occur on the lining of the cheek and under the tongue (Greenspan et al, 1984) The lesions are white in appearance with an irregular surface and fine vertical corrugations. (Greenspan et al, 1984, Kabani et al 1989). The size and appearance of the lesion varies and it can be unilateral or bilateral. Most commonly OHL is asymptomatic

and does not require treatment. Lesions regress with prolonged acyclovir treatment and temporary resolution has been achieved with desciclovir, an analog of acyclovir (Greenspan et al, 1990).

The lesion contains replicating EB viral DNA and may be co-infected with multiple EBV strains (Webster-Cyriaqu & Raab-Traub, 1998). Webster-Cyriaqu & Raab-Traub characterized EBV gene transcription within OHL biopsy specimens and found expression of EBERS 1 & 2, EBNA1-6, LMP1, LMP 2a and 2b and BamHI A rightward reading frame mRNA in the lesion (Webster-Cyriaqu & Raab-Traub, 1998).

1.6 Cytokines Production by EBV Infected Cells

Cytokines are soluble proteins and peptides which act as humoral regulators at the inter and intra-cellular level to modulate functional activities of individual cells and tissues both under normal and pathological conditions (Balkwill & Burke, 1989). Effects of cytokines can be autocrine, paracrine, or both (Kennedy and Jones, 1991). Unlike classical hormones, cytokines are not produced by cells present in specialized glandular tissue, and they act on a wider spectrum of target cells (Imura et al, 1991). Cytokine network is a term used to refer to the interactions between cytokines to control synthesis and biological activities of themselves or/and other cytokines (Balkwill and Burke, 1989). The cytokine network usually operates in a cascade where one cytokine triggers the expression of one or more cytokines, which in turn, induce production or inhibition of other cytokines or growth factors. Factors produced earlier or later in the cascade may affect other cytokines and create feedback regulatory circuits. Cytokines can have both beneficial and damaging effects depending upon the pathology which stimulates or inhibits their secretion.

Studies have shown that addition of monocytes to *in vitro* cultured EBV infected B cells can potentiate their growth and transformation (Tosato et al, 1988). Cell free supernatants from cultured monocytes and EBV immortalized B cells have also been shown to promote growth of EBV transformed cells *in vitro* (Tosato et al, 1989). These findings suggest that EBV transformed cells produce soluble B

cell growth factors (cytokines), which are released in the supernatant and can promote their own growth. In contrast, it has also been reported that T cells inhibit the growth of EBV infected cells both by cytotoxicity and production of growth supressing cytokines (Rickinson et al, 1996). This data suggests that the growth of EBV infected B cells is controlled through both autocrine and paracrine production of cytokines. Autocrine cytokine production by EBV infected B cells is influenced by LMP1 via its ability to activate NF-κB/REL transcription factors (Huen et al, 1995; Devergne et al, 1996). These transcription factors exist in an inactivated form in the cytosol, complexed with a group of inhibitory IκB proteins (Siebenlist et al, 1994). When activated they regulate the expression of IL-1, 2, 6, 8, tumour necrosis factor (TNF) α and TNFβ (Siebenlist et al, 1994).

1.6.1 IL-1

Both translated IL-1 protein and transcribed IL-1 mRNA have been detected in LCLs *in vitro* in several studies (Scala et al, 1984; Matsushima et al, 1985; Vandenabeele et al, 1990). IL-1 secreted by LCLs is functional and increases cellular proliferation rate (Blazar et al, 1990). IL-1 up-regulation is mediated mainly by LMP1 through activation of NF-κB (Krauer et al, 1998).

1.6.2 IL-2

IL-2, also called the T cell growth factor (TCGF), induces non-antigen related proliferation of T cells. Due to its positive effects on T cell proliferation IL-2 has been used in adoptive immunotherapy of various human cancers (discussed in section 1.5.1.3.2). Previous studies have shown that IL-2 is also secreted by LCLs (Mouzaki et al, 1995) and normal B cells can be induced to secrete IL-2 if stimulated with CD40 ligand (Kindler et al, 1995). IL-2 also promotes proliferation of activated B-cells and both normal and malignant B cells express an IL-2 receptor, which suggests that IL-2 can act on B cells in an autocrine fashion (de Totero et al, 1995). IL-2 stimulates IFNγ production in peripheral lymphocytes and also induces the secretion of IL-1 , TNF-alpha and TNF-beta .

1.6.3 IL-4

Banchereau et al reported IL-4 as a B cell growth factor irrespective of EBV infection (Banchereau et al, 1991). In their study IL-4 promoted long term *in vitro* growth of EBV negative B cells. A recent study has reported that IL-4 enhances B cell proliferation by reducing the average time cells take to enter the first division cycle and by promoting normal B cell survival (Hasbold et al, 1999). In contrast, it has been reported that EBV immortalized B cells produce IL-4 at a very low level (Ohnishi et al, 1997). Other studies have shown that IL-4 either has no effect on growth of LCLs and may actually inhibit their proliferation (Bende et al, 1992; Defrance et al, 1992). It has been suggested that inhibition of B cell growth by IL-4 is due to its ability to inhibit IL-6 synthesis, which is a B cell growth factor (Hermann et al, 1991).

1.6.4 IL-6

IL-6 is recognised as a B cell growth and differentiation factor (Yokoi et al, 1990). The main source of IL-6 *in vivo* is activated monocytes, fibroblasts and endothelial cells (Helle et al, 1991). Feeder layers of activated monocytes and fibroblasts have been shown to promote activation and proliferation of B cells *in vitro* and IL-6 could be isolated from the culture supernatants (Tosato et al, 1988). IL-6 is produced by LCLs (Tosato et al, 1990) and it has been reported that these cells express a functional IL-6 receptor. This suggests that EBV infected B cells might maintain their own growth by autocrine production of IL-6. Other studies have shown that LCLs transfected with IL-6 gene are more tumourigenic than non-IL-6 transfected LCLs in athymic mice (Scala et al, 1990; Tanner et al, 1991). Increased tumourigenicity of IL-6 is thought to be because of its effects on B cell growth and differentiation (Yokoi et al, 1990), its ability to inhibit NK cell activity (Tanner et al, 1991) and its angiogenetic properties (Folkman, 1992).

1.6.5 IL-10

IL-10 is thought to be an immunosupressive cytokine. Transgenic mice expressing human IL-10 fail to mount a significant T or B cell immune response when immunologically challenged (Groux et al, 1999). IL-10 is produced *in vitro* by both normal and EBV-transformed B cells (Armitage et al, 1993; Burdin et al,

1993; Sairenji et al, 1998). IL-10 inhibits the synthesis of IFNγ by Th-1 cells (D'Andrea et al, 1993) and this inhibition is mediated indirectly by suppression of IL-12 production (Gagro & Gordon, 1999). IL-10 is also known as B cell differentiation factor (BCDF) and *in vitro* stimulates B-cell differentiation, increases the MHC class II expression on these cells and induces antibody production in activated B cells (Emelie et al, 1997). A previous study has reported that IL-10 abrogates the cytotoxic capacity of T cells through suppression of T-cell activation and through enhancement of growth of EBV-infected B-cells (Bejarano & Masucci, 1998). Addition of exogenous IL-10 and EB viral preparation to cultured B cells potentiates virus induced B cell transformation and proliferation (Burdin et al, 1993). Other studies have observed a reduction in cellular proliferation on addition of neutralizing anti-IL-10 antibodies added to B cell cultures and LCLs (Stuart et al, 1995; Beatty et al, 1997). IL-10 has similar effects on B cells as IL-6 and a recent study investigating their interaction has reported that although both IL-10 and IL-6 promote B cell differentiation and Ig production, they do so by independant mechanisms (Bonig et al, 1998). They showed that stimulation of B-cells with IL-10 suppressed autocrine IL-6 production. However, IL-6 levels remained sufficiently high to stimulate its receptor, and IL-6 binding to the B-cell surface was not affected (Bonig et al, 1998).

1.6.6 IFN-γ

IFN-γ is predominantly a negative regulator of B cell differentiation and proliferation (O'Neil et al, 1999). *In vitro*, it upregulates the interaction between T cells and APCs by inducing expression of costimulatory molecules such as CD80 and CD86 on lymphoma and leukaemia cell lines (Tsukada et al, 1997; Zheng et al, 1998). IFN-γ has also been reported to arrest tumour growth in conjunction with CD8+ T cells in tumour mouse models (Farrar et al, 1999) and in a study by Sgadari et al, it caused regression of subcutaneous human BL tumours in nude mice (Sgadari et al, 1997).

1.6.7 Viral IL-10 (vIL-10)

EB viral BCRF-1 protein is expressed during the lytic phase of EBV life cycle in B-lymphocytes (Stewart et al, 1994) but not in latency and therefore is thought not to play a role in pathogenesis of EBV related malignancies (Hayes et al, 1999). It is structurally related and functionally similar to human IL-10 and is termed viral IL-10 (vIL-10) (Hsu et al, 1990; Vieira et al, 1991). vIL-10 can be detected in the serum in 50-60% cases of acute IM and chronic active EBV infection, (Taga et al, 1995; Kanegane et al, 1997) and it is suggested that vIL-10 suppresses antiviral immune responses by specifically inhibiting T cell proliferation in response to antigenic stimuli (Waal Malefyt et al, 1991). T cell inhibition by vIL-10 is mediated by suppression of IFNγ production (Vieira et al, 1991) and down-regulation of cytokine production by monocytes (de Waal Malefyt et al, 1991). Like human IL-10, vIL-10 promotes B cell proliferation and prevents apoptosis in these cells (Rousset et al, 1992; Burdin et al, 1993; Levy et al, 1994). Miyazaki et al have reported that blocking of vIL-10 gene with antisense oligonucleotides resulted in inhibition of transformation of B cells (Miyazaki et al, 1993). These reports suggests that like human IL-10, vIL-10 promotes the growth and proliferation of EBV infected B cells.

1.7 Cytokine Production by EBV Related Malignancies

1.7.1 PTLD

The cytokine profile of PTLD tumours has been studied by serological, reverse transcriptase polymerase chain reaction (RT-PCR) and flowcytometry techniques. In PTLD patients serum levels of IL-4, 6 and 10 have been reported to be raised as compared to normal controls (Tosato et al, 1993; Mathur et al, 1994; Garnier, 1999) and IL-4 and IL-10 mRNA has been detected in primary PTLD tissue by RT-PCR (Nalesnik et al, 1999). Johannessen et al, detected IL-2, 4, 6, 10 and INFγ mRNA by RT-PCR in EBV positive PBMC induced I/P tumours in SCID mice, which serve as an *in vivo* animal model for PTLD (Johannesson et al, 2000). PTLD tumours have been shown to be heavily infiltrated with CD4-positive T cells (Perera et al, 1998) and it is possible that apart from autocrine cytokine production by tumour cells, infiltrating T cells may be another source of cytokine

production in PTLD. Birkeland et al, compared pre- and post-transplant serum levels of IL-10 from PTLD patients and have reported a progressive increase in serum IL-10 levels with disease progression (Birkeland et al, 1999).

1.7.2 Hodgkin's Disease (HD)

Blay et al measured serum levels of IL-1 alpha, IL-2, IL-4, IL-6 and tumour necrosis factor (TNF) in 24 untreated patients with Hodgkin's disease by ELISA and detected increased levels of IL-1, IL-2 and IL-6 in most cases compared to healthy controls (Blay et al, 1994). In another study the expression of IL-10 in HD tissue was investigated in 30 cases by immunostaining. Hodgkin and R/S cells in 16 of the 30 cases were EBV positive and tested positive for IL-10 expression (Ohshima et al, 1995). Studies by other groups have also confirmed the expression of IL-10 in HD (Herbst et al, 1996) and high serum levels of IL-10 have been correlated with poor prognosis (Sarris et al, 1999). Increased IL-6 gene expression and serum IL-6 levels have also been reported in HD (Gruss et al, 1992; Kurzrock et al, 1993). Herbst et al, investigated IL-6 expression in EBV positive and negative cases of HD. 84% of the EBV positive cases expressed IL-6 while only 51% of the EBV negative cases expressed IL-6 (Herbst et al, 1997). Like IL-10, serum IL-6 levels are also thought to be correlated with clinical outcome of the disease, with IL-6 levels rising before treatment is started and returning to undetectable once remission has been acheived (Seymour et al, 1997).

1.7.3 Nasopharyngeal Carcinoma (NPC)

A recent study has reported immunohistochemical detection of increased IFN-γ levels in EBV related NPC (Tang et al, 1999). In this study the IFN-γ signal was predominantly observed in the infiltrating T lymphocytes. Fujieda et al, detected IL-10 expression in EBV-related NPC by immunohistochemistry and they negatively correlated its expression with 5 year survival of the patients (Fujieda et al, 1999). The study included 21 patients out of which 12 tested positive for IL-10. 87.5% of the IL-10 negative group survived for 5 years in contrast to only 15.6% of IL-10 positive patients. Statistical analysis showed that IL-10 expression was significant as an independent prognostic indicator of overall survival (Fujieda et al, 1999). Huang et al, recently investigated the expression profile of 12

cytokines in NPC tumours by flowcytometry. RT-PCR was used to detect IL-1, 2, 4, 5, 6, 10, INFγ, TNFα, TGFβ, and IL-1 receptors. The tumours tested positive for all cytokines with elevated expression of IL-1 compared to control tissue. They identified the cell types responsible for IL-1 production to be both the malignant epithelial cells and the infiltrating CD4+ T cells (Huang et al, 1999).

1.8 The Severe Combined Imuunodeficient (SCID) Mouse Model

SCID mice are C.B.-17 strain mice, which lack functional T and B cells (Bosma et al, 1983). They harbor the *scid/scid* mutation, which affects the V(D)J recombinase enzyme system involved in immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements (Schuler et al, 1986). The *scid/scid* mutation prevents productive rearrangement of immune receptor genes and results in arrest of developing lymphoid cells at the immature "triple negative" phase, which do not express T and B cell surface markers (Murphy et al, 1994). Although transcripts of TCR γ and β have been detected in SCID thymocytes, transcripts of complete TCR gene rearrangements have not been demonstrated (Schuler et al, 1986). Similarly complete transcripts of Ig gene rearrangements have not been detected in immature SCID B cells (Schuler et al, 1988). With maturation, SCID mice develop rudimentary antibody and T cell responses and are referred to as "leaky" (Bosma, 1991). By 12 months of age SCID mice develop low levels of antibody and oligoclonal B cells and T cells (Bosma, 1992). Some of the T cells are functional because they can cause rejection of allogeneic skin grafts but do not proliferate in response to lymphocyte mitogens (Bosma et al, 1992).

1.8.1 Xenograft of Human PBMC in the SCID Mouse; Model for EBV-Related Lymphoid Malignancies

Due to absence of functional T and B cells, SCID mice accept xenografted human cells. Transplanted human PBMC in the SCID mouse survive for at least 13 months and also maintain their immune function (Mosier et al, 1988, 1989). This property makes these animals an ideal model to study human viral infections such as HIV (van Kuyk et al, 1994) and cancers such as metastatic melanoma (Sabzevari et al, 1993) and post-transplant lymphoma (Boyle et al, 1993). When SCID mice are injected I/P with PBMC from EBV seropositive donors some

develop tumours within 8-16 weeks (Mosier et al, 1988, 1991; Ramqvist et al, 1991). The tumours are of human B cell origin and histologically resemble immunoblastic lymphomas (Mosier et al, 1988, 1990). The tumours cells have the same surface phenotype as LCL cells and express CD 19, 20, 23, 39, and HLA Class II molecules (Mosier et al, 1990). The cells express EBNA 1, 2, 3, 4, 5 and 6 and LMP1 & 2 (Rowe et al, 1990), which is consistent with type III EBV latent gene expression pattern and is similar to EBV gene expression in the majority of PTLD tumours (section 1.5.1.2). Thus these tumours are a model for studying PTLD pathogenesis and investigating the efficacy of new treatment modalities *in vivo*. Although some studies have reported that human CD8+ T cells show diminished function when injected into SCID mice (de Kroon et al, 1997), tumours induced in these animals by I/P or I/V injection of human LCL or PBMC from EBV seropositive donors have been successfully treated with adoptive transfer of *in vitro* generated autologous EBV-specific T cells (Boyle et al, 1993; Buchsbaum et al, 1996). Boyle et al, generated EBV-specific CTL by *in vitro* stimulation of PBMC with autologous irradiated LCLs. SCID mice were injected with LCL cells and EBV-specific CTLs were either adoptively transferred immediately or seven days after LCL injections. Control groups included SCID mice engrafted with LCL cells alone. Animals in the control group rapidly developed B cell lymphomas (mean, 20 days). However, tumours were either prevented or significantly delayed in mice injected with LCL and CD8+ EBV-specific CTLs. In contrast, HLA mismatched EBV-specific CTL failed to suppress or delay tumour occurence. (Boyle et al, 1993). In another study Lacerda et al, demonstrated that after CTL infusion, the PBMC-induced SCID tumours showed infiltration of T cells into the tumour and cause rapid tumour regression (Lacerda et al, 1996). They initially tested the effects of adoptive transfer of autologous PBMC, purified T cells, interleukin IL-2-activated PBMC or anti-CD3-activated T cells on PBMC induced tumours in SCID mice. None of the above cell types improved survival of treated mice. In contrast, *in vitro* expanded EBV-specific CTL, from EBV-seropositive donors exhibited strong EBV-specific and HLA-restricted activity both *in vitro* and *in vivo* and caused complete regression of autologous PBMC induced tumours. Moreover, these CTLs did not have any effect on HLA-mismatched LCL induced tumours. Mice bearing two

subcutaneous EBV+ tumours, one autologous and the other HLA mismatched to the EBV-specific CTL donor, showed regression of only the autologous tumour after intravenous infusion of EBV-specific CTLs. Autologous CTLs were detectable in the tumours 24 hours after intravenous adoptive transfer and immunophenotypic analyses showed the infiltrating cells to be predominantly CD3 and CD8 positive and CD4 negative (Lacerda et al, 1996).

1.9 Aim of the Study

The present study was divided into two parts. The aim of the first part was to investigate the *in vivo* effect of different immune effector cells on outgrowth of autologous EBV positive human tumours in the SCID mouse. The second part of the study focused on the cytokine gene expression pattern of EBV-associated diseases, the cell type producing the cytokines and relationship between EBV infection and cytokine expression by non-isotopic in situ hybridisation technique.

2. Materials and Methods

2.1 Chemical & Reagents

Chemical/Reagent	Cat. No.	Company
Acetone	100034Q	BDH
BSA	A3059	Sigma
DEPC	D5758	Sigma
DMSO	D8418	Sigma
EDTA	E7889	Sigma
Ethanol molecular biology grade	43743 4U	BDH
Evans blue	34115 2L	BDH
Ficoll	F2637	Sigma
Formalin	F1635	Sigma
Formamide	F/P565/PB08	Fisher
Giemsa stain	35086 4X	BDH
Glycerol	284545E	BDH
Hydrochloric acid	101254H	BDH
iso-Pentane	103614T	BDH
Magnesium chloride	M2670	Sigma
Magnesium sulphate	M2643	Sigma
Silane	M6514	Sigma
Mix bed resin	M8032	Sigma
Paraformaldehyde	294474L	BDH
PBS	BR1La	SLS
Phosphate citrate buffer	P4809	Sigma
Polyvinylpyrrolidine	P5288	Sigma
Potassium chloride	P/4280/53	Fisher
Potassium dihydrogen orthophosphate	60353	Fluka
Potassium-di hydrogen orthophosphate	P/5245/60	Fisher
RNase	R-5503	Sigma
RNase away	73129	Kramel Biotec
Sodium azide	30111 2G	BDH
Sodium carbonate	10240 4H	BDH

Chemical/Reagent	Cat. No.	Company
Sodium chloride	S7653	Sigma
Sodium citrate	S/3280/65	Fisher
Sodium hydrogen carbonate	10247 4V	BDH
Sodium hydroxide pellets	S/4880/60	Fisher
Sodium-di hydrogen orthophosphate	30158 4L	BDH
Sulphuric acid 2.5M	191675A	BDH
Triton X100	X-100	Sigma
Trizma base	103157P	BDH
Xylene	102936H	BDH

2.2 Laboratory Equipment

Product	Supplier	Make
Balance	Phillip Harris	Sartorius
Carousel	Scotlab/Anachem	Gilson
Cell harvester	Skatron	Skatron
Centrifuge	Fisher	Sanyo
Centrifuge: microfuge	SLS	MSE
^{137} Cesium Source	Nordion	Canadian Atomic Energy
Cytospin	Shandon	Hybaid
Forceps: dissecting	Inter Focus	BDH
Freezer -20^{O}C	John Lewis	Liebher
Freezer -70^{O}C	Cryotechnics	Assab
Fridge	Wolf Labs	Labcold
Gamma counter/printer	EG&G Wallac	Wallac
Haemocytometer	BDH	BDH
Immunostaining tray	RA Lamb	RA Lamb
Incubator CO_2	Leec	Leec
Liquid nitrogen container	Boro Labs	L'Air Liquid
Lquid nitrogen dewar	Merck	Dilvac
Lquid nitrogen dewar 25L	Cryotenics	Statebourn
Magnetic stirrer	Jencons	Voss

Product	Supplier	Make
Microbiological saftey cabinet	MAT	MAT
Micropipettes	Life Science	Gilson
Microscope: binocular	Leitz	Leitz
Microscope: inverted	R A Lamb	Olympus
Multichannel Pipette	Life Science Int	Finnpipette
pH meter	Fisher	Hanna
Pipette aid	SLS	Bibby
Radiation: mini Viewing Barrier	Nuclear Services	Nuclear Services
Radiation: monitor & case	Mini Instruments	Mini Instruments
Reflector and stand	Int Market Supply	BDH
Rocking table	Denley	Lukham
Stirrer/hotplate	Merck	Stuart Scentific
Thermometers	BDH	BDH
Vortex	Merck	Genie
Waterbath	Merck	Grant

2.3 Suppliers Contact Information

Aldrich Chemical Co Ltd, Gillingham, Dorset

Amersham / Pharmacia Biotec Uk Ltd, Little Chalfont, Buck

Anachem Ltd/ Scotlab, Luton, Bedfordshire

BDH/Merck Ltd, Box 15, Fresh Water Rd, Dagenham, Essex

Becton Dickinson, Between Towns Rd, Cowley, Oxford

Bibby Sterilin, Tilling Drive, Stone, Staffordshire

J. Bibby Science Products, Tilling Drive, Stone, Staffs

Boc Ltd Special Gases, Deer Park Rd, London

Boehringer Mannheim Uk Ltd, Bell Lane, Lewes, East Sussex

Boro Labs, Paices Hill, Aldermaston, Berks

Bright Technologies Ltd, Kilamarsh, Sheffield

Cambridge Bioscience, Newmarket Rd, Cambridge

Camlab Ltd, Nuffield Rd, Cambridge

Chemicon International Inc., Cricklewood Lane, London

Chiron Uk Ltd, Salamander Quay West, Park Lane, Harefield, Middlesex

Corning Costar Uk, High Wycombe, Bucks

Cryotechnics, Albion Road, Edinburgh

Dako Ltd, Denmark House, Angal Drove, Ely, Cambridge

Denley Instruments Ltd, Denley House, Hawkin Rd, Colchester

EG&G Wallac, Crownhill Business Centre, Crownhill, Milton Keynes

European Instruments, Headington, Oxford

Farenheit Lab Supplies, Rotherham, Yorks

Fisher Scientific UK, Loughborough, Leicestershire

Gibco/Life Technologies Ltd, Inchinnan Buisness Park, Paisley

C A Hendley (Essex) Ltd, Loughton, Essex

Heraeus Equipment, 9 Wates Way, Brentwood, Essex

Hybaid Ltd, Ashford, Middlesex

ICN Biomedical, Basingstoke, Hampshire

ICN Pharmaceuticals, Thame, Oxon

Interfocus, Haverhill, Suffolk

Invitrogen, De Schelp 12, 9351 Nv Leek, Holland

Jencons Scienific Ltd, Leighton Buzzard, Bedfordshire

Kimberley Clark Ltd, Larkfield, Maidstone, Kent

Life Science International (Uk) Ltd, Basingstoke, Hampshire

Microinstruments (Oxford) Ltd, Witney, Oxford

Millipore Ltd, Watford, Hertfordshire

Mini-Instruments Ltd, Burnham on Crouch, Essex

Nuclear Services Ltd, Bude, Cornwal

Olympus Optical Uk Ltd, Honduras St, London

Oxoid Ltd, Basingstoke, Hants,

Philip Harris Scientific, Park Royal, London

Promega, Delta House, Chilworth Research Centre, Southampton

Raymond Lamb, London

R & D Systems Europe Ltd, Abingdon, Oxofordshire,

Sanyo Gallenkamp Plc, Monarch Way, Loughborough, Leicestershire

Scientific Lab Supplies Ltd, Wilford, Nottinghamshire

Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire

Serotec Ltd, Kidlington, Oxfordshire

Sigma Chemical Co. Ltd, Poole, Dorset

Tissue Culture Services Ltd, Claydon, Buckingham

Vector Labs, Bretton, Peterborough

Whatman Lab Sales Ltd, Maidstone, Kent

Wolf Laboratories Ltd, High Wycombe, Buckinghamshire

2.4 Solutions and Reagents

2.4.1 Cell Culture

NOTE: ***All reagents stored at +4^{0}C unless stated otherwise.***

Culture Medium

(All ingredients stored at -20^{O}C)

L-Glutamine	2mM
Penicillin	100U/ml
Streptomycin	100ug/ml
Amphoterecin B	0.5ug/ml
Fetal Calf Serum	10-20% v/v

Made up in 1xRPMI

Wash Medium

Hanks Balanced Salt Solution (HBSS)

Freezing Medium

Fetal Calf Serum	90% v/v
DMSO	10% v/v

Interleukin 2 (IL-2)

Stock concentration: 10U/ul

Dissolved in dH_2O and stored at -20^{0}C in 50ul aliquots.

Phytohaemagglutinin (PHA)

Stock concentration	500ug/ml

Made in PBS and stored at -20^{0}C

2.4.2 In Situ Hybridization

NOTE: ***All reagents made up in DEPC treated ddH$_2$O, autoclaved (except those containing alcohol) prior to use and stored at R/T unless stated otherwise.***

DEPC Treated ddH$_2$O

DEPC	0.5% v/v

Stirred and kept overnight and autoclaved.

Ethanol

In DEPC water	99%, 95%v/v

1xTBS pH7.6

Tris-HCl	0.05M
NaCl	0.15M

Made in ddH$_2$O.

10xTBS

Tris-HCl	0.5M
NaCl	1.5M
$MgCl_2$	0.02M
BSA	1% v/v

Made in ddH$_2$O.

Modified TBS

BSA	0.1% v/v
$MgCl_2$	0.02M
Triton X-100	0.1% v/v

Stored at 4^{0}C

20xSSC

NaCl	3M
Na_3Citrate	0.3M

Deionised Formamide

Formamide plus AG 501-X8 Resin	10% w/v

Stirred over night in fume hood and filtered.

Ribonuclease A (RNase) Buffer

NaCl	500 mM
Tris-HCl	10 mM
EDTA	1 mM

10x Phosphate EDTA (PE)

Tris pH 7.6	0.5M
PVP	0.05mM
Di-sodium pyrophosphate	0.04M
Ficol	0.05mM
EDTA pH 8	0.05M

Stored at 4^0C

Hybridization and Pre-Hybridization Solution

NaCl	0.6M
Dextran Sulphate	0.2mM
Formamide (deionised)	6.6M
x10 PE	1% v/v
SSD	2.2mg

Stored at -20^0C

Paraformaldehyde

(Made in PBS)	0.4% w/v

Made fresh with each run.

Proteinase K

Diluted in 5mM Tris-HCl — 10ug/ml

Stored at -20^{0}C

4% Formalin

(Stock concentration: 37% v/v) — 5.4% v/v

Made in PBS.

PBS:Glycerol — 50% v/v

Made in PBS

Citrate Buffer

Sodium citrate — 0.6% w/v

Giemsa Stain — 5% v/v

Animal Sera — 2-20% v/v

(Diluted in Modified TBS) Stored at -20^{0}C

Silane (Made in Acetone) — 2% v/v

2.5 Probe Cocktails for in situ detection of cytokine mRNA

Antisense oligonucleotide probe cocktails were obtained from R&D systems, UK. Each probe cocktail contained equimolar mixtures of sequences from within 3-4 exons from each cytokine mRNA sequence. The average length/number of base pairs per exon was 30 and each was digoxigenin (DIG) labelled at both 3' and 5' ends. Probes were dissolved in 250µls of DEPC treated water to give a stock concentration of 20ng/µl and stored at -70^{O}C in 5ul aliquots. For use 45µls of hybridization buffer was added to each 5µl aliquot to get a final concentration of 2ng/µl. 20ng of probe was added to each section during in situ hybridization procedure. The probes and their properties are listed in table 2.1

Table 2.1

Oligonucleotide Probe Cocktails used for In Situ Detection of Cytokine mRNA

Probe	Exons (equimolar mixture)
IL-2	1, 2, 3, 4
IL-4	5', Mid and 3' Regions
IL-6	2, 3, 4, 5
Il-10	-
INFγ	1, 2, 3
β-actin	3, 4, 5, 5/6

2.6 Primary, Secondary and Tertiary Antibodies and Normal Animal Sera

Details of primary, secondary and tertiary antibodies used in this study are listed in tables 2.2 and 2.3. Primary antibodies were titrated individually to establish the optimum concentration required for positive results. Secondary and tertiary antibodies were used at concentrations ranging between 1:100 to 1:1000 (as suggested by manufacturer). Normal animal sera were filtered and stored at -20^{0}C.

Table 2.2

Primary Antibodies used in the Study

Antibody	Reactivity	Host	Clone	Isotype	Dilution Used
CD20	Human	Mouse	L-26	IgG2a,kappa	1:50
CD15	Human	Mouse	MY-1	IgM, kappa	1:50
CD30 (Ki-1)	Human	Mouse	Ber-H2	IgG1	1:50
CD20	Human	Mouse	BCA-B/20	IgG2a	1:20
CD57	Human	Mouse	NK-1	IgM	1:20
Macrophage	Human	Mouse	LN-5	IgM	1:20
CD3	Human	Rabbit	Polyclonal	IgG	1:20
CD3	Human	Mouse	Polyclonal	IgG	1:10
CD8	Human	Mouse	Polyclonal	IgG	1:10
DIG	DIG	Sheep	Polyclonal	IgG	1:25
FITC	FITC	Rabbit	Polyclonal	IgG	1:100

DIG: Digoxigenin, FITC: Fluorecein isothiocyanate,

Table 2.3

Secondary & Tertiary Antibodies used in the Study

Specificity	Host	Isotype	Reporter Molecule
Mouse	Rabbit	IgG	Biotin
Mouse	Rabbit	IgG	FITC
Sheep	Rabbit	IgG	Biotin
Rabbit	Goat	IgG	Biotin
Sheep	Rabbit	IgG	Biotin
Goat/Sheep	Donkey	IgG	FITC
Digoxigenin	Sheep	IgG	AP
Mouse	Sheep	IgG	FITC
Mouse	Sheep	IgG	HRP
Mouse	Sheep	IgG	Biotin
Mouse	Sheep	IgG	FITC
Rabbit	Donkey	IgG	Biotin
Goat/Sheep	Donkey	IgG	Biotin

FITC: Fluorecein isothiocyanate, AP: Alkaline phosphatase, HRP: Horseradish peroxidase

2.7 Cell Culture Techniques

2.7.1 Culture Conditions

Cells suspended in culture medium (CM) were either plated out in 24 and 96 well plates or in $25cm^2$ or $75cm^2$ cell culture flasks at 37° C in 5% CO_2 and fed as required.

2.7.2 Feeding of Cell Lines

Cell lines kept in 24 or 96 well plates were fed weekly or as required by replacing half of the medium from each well with fresh CM. Cultures kept in flasks were fed by addition of fresh CM as required.

2.7.3 Thawing and Washing of Cell Lines

Frozen cell lines were thawed in a water bath at 37°C. Hank's balanced salt solution (HBSS) (wash medium) was added slowly to prevent cell disruption. Cells were centrifuged for seven minutes at 400g. The supernatant was discarded and the cell pellet was resuspended in CM.

2.7.4 Cell Count and Viability

Cells were counted in a Neubaur haemocytometer by making a 1:1 dilution of cell suspension in trypan blue (0.5% w/v). Viability of cells was checked by not counting cells staining with Trypan blue (non-viable cells).

2.7.5 Viable Freezing of cell lines

Cells were counted, washed once and frozen in (FM) at a cell concentration of 2-5 x 10^7 per 1ml of medium. Cells were transferred to a 1.5ml cryotube and stored at -70^{O}C for 24 hours before transfer to liquid nitrogen.

2.7.6 Separation of Peripheral Blood mononuclear Cells (PBMCs) from Heparin treated Whole Blood

Heparinized whole blood was layered over an equal volume of Ficol-Hypaque^{TM} in a 50ml universal tube and centrifuged at 540g for 15 minutes. Red cells sedimented to the bottom of the tube and PBMCs formed a layer at the interface between the Ficol-Hypaque^{TM} and plasma. 500uls of plasma were collected in a cryotube and kept at 4^{O}C for EBV serology. The PBMCs were harvested, washed twice, and either cultured or frozen for future use.

2.7.7 Phytohaemagglutinin (PHA) Stimulation of PBMC

T cell growth was stimulated *in vitro* by addition of 10ug/ml of PHA to PBMC at a concentration of 2 x 10^6/ml in CM. Cells were kept at 37^{0}C in 5% CO_2 for 48 hours, then washed and used as required.

2.7.8 *In vitro* generation of LCLs

10^7 washed PBMCs were suspended in 1ml of concentrated EBV preparation (kindly provided by Professor D H Crawford, Medical Microbiology, University of Edinburgh) and incubated at 37^{O}C in 5% CO_2 for one hour. Cells were washed twice to remove unbound virus, suspended in 10mls of culture medium containing cyclosporin A (CyA) and plated out in 5 wells of a 24 well plate at a final cell count of 2 x 10^6 cells in a volume of 2mls/well. Cells were incubated at 37^{O}C in 5% CO_2 and fed weekly. After 4-6 weeks cell viability was checked by trypan

blue exclusion. Cells were harvested, transferred to a $25cm^2$ cell culture flask and expanded and/or frozen as required.

2.7.9 γ-Irradiation of Cells

Cell proliferation was inhibited by introduction of DNA damage by exposure to γ-irradiation from a ^{137}Cesium source. Exposure time (ET) required to deliver 4000 rads was calculated by the following formula:

$$\mathbf{ET = \frac{Y \times 60 minutes}{4.07 \times 10^4 \text{ rad/hour} \times DF} \times 1.10}$$

Y was the desired irradiation dose (4000 rads for LCLs and 2000 rads for PBMCs) and DF was decay factor for the cesium source.

2.7.10 *In vitro* Cytotoxic T Cell Culture

PBMCs were plated in a 24 well plate at a concentration of 2×10^6 cells/well (in 1ml of CM). 5×10^4 irradiated autologous LCL cells suspended in 1ml of CM were added to each well (PBMC:LCL ratio of 40:1) to stimulate EBV-specific CTL growth. Cells were incubated for ten days at 37°C in 5% CO_2 and observed under an inverted microscope for CTL clumps. On day 10 cells were harvested, pooled, counted and re-stimulated with irradiated autologous LCL at a ratio of 4:1 (10^6 CTL plus 5×10^4 LCL cells in 2mls of CM/well) in a 24 well plate. On day 14, 40 units of recombinant human IL-2, in 100uls of CM were added to each well. CTL cultures were maintained and expanded by addition of IL-2 on every second day and weekly re-stimulation with irradiated autologous LCLs at CTL:LCL ratio of 4:1. Cells were frozen once sufficient numbers had been generated.

2.7.11 *In vitro* Generation of Peptide Specific CTLs

Individual aliquots of PBMC were pulsed for two hours at 37°C with exogenous peptides at a concentration of $20ug/10^6$ cells. Peptide-pulsed PBMC were washed once, irradiated at 2000 rads and were mixed with fresh autologous PBMC at a ratio of 1:1. Cells were cultured for ten days in 24 well culture plates at a concentration of $2x10^6$ cells in 2mls of CM/well. On day 10 the cells were re-

stimulated with peptide labelled, irradiated autologous PBMC at a ratio of 1:1 and kept in culture in a 24 well plate for a further four days. On day 14 cells were either frozen or used as required.

2.7.12 Limiting Dilution Analysis

Precursor frequency for EBV-specific CTLs in PBMC was measured using the limiting dilution analysis. 20,000 responder cells (CTLs) in 200uls of CM were added to wells in the first row of a 96 well round-bottomed plate. 100uls of CM was added to the next five rows. Serial dilutions of responder cells were made by taking 100uls from the first row and adding it to the second row. The process was repeated for all rows. Six to twelve replicate wells were set up for each cell concentration. $2.5x10^4$ peptide coated and irradiated stimulator cells (autologous PBMC) in 100uls of CM were added to each well. The cells were cultured for 14 days at $37^{O}C$ in 5% CO_2 to allow differentiation and expansion of CTL precursors. On day 14 the contents of each well were harvested and tested against peptide-pulsed autologous PBMC in a standard ^{51}Cr release assay. Wells were scored as positive if the precent release of ^{51}Cr exceeded the mean release from the non-stimulated spontaneous release control wells. The number of cultures that failed to lyse the peptide-labelled PBMC was recorded for each set of replicates. Poisson distribution was used to determine the concentration of cells in a well that on average, contained a single, specific precursor cell.

2.7.13 Cytotoxicity Assay on Polyclonal and Peptide-Specific CTL Lines

Cytotoxicity of polyclonal and peptide-specific CTLs was tested in a standard four hour ^{51}Cr release assay. Targets for polyclonal CTLs included autologous LCL cells, allogeneic LCL cells and K562 (NK target) cells. Targets for peptide-specific CTLs included autologous peptide labelled and unlabelled PBMC, autologous LCL and allogeneic LCL. Cytotoxicity of each CTL line was tested at 3 cell concentrations ($2x10^5$, 10^5 and $5x10^4$) at CTL:LCL ratios of 20:1, 10:1 and 5:1. Each cell concentration was plated in triplicate in a 96 well round bottomed plate in a final volume of 100ul of CM/well. CTLs were plated out in separate plates for each target cell line. Triplicate maximum release wells containing

100uls of 1.0% Triton-X 100, and spontaneous release wells containing 100uls of CM were included as controls.

10^6 cells from each target were incubated with 3.7 MBq of ^{51}Cr for one hour at 37^0C. Cells were washed twice and re-suspended in 10mls of CM to give a final cell concentration of 10^5 cells/ml. 100uls (10^4 cells) of the target cell were added to spontaneous and maximum release wells and to all wells containing CTL. Cells were incubated at 37^0C for 4 hours or overnight (16 hours). Following the incubation period, plates were centrifuged at 500g for 7 minutes and 100uls of supernatant from each well was transferred to scintillation vials and read in a gamma counter. Counts per minute were recorded for each well. All triplicates were averaged and percent-specific lysis was calculated using the following formula.

$$\% \text{ Specific Lysis} = \frac{\text{Test Value - Spontaneous Release}}{\text{Maximum Release - Spontaneous Release}} \times 100$$

2.8 *In vivo* Effect of Autologous CTL on Outgrowth of LCL Induced Subcutaneous Tumours In SCID Mice

2.8.1 SCID Mice

3-6 weeks old SCID mice were obtained from the transgenic unit of Medical Microbiology, University of Edinburgh. Animals were kept under aseptic conditions in isolators.

2.8.2 Definition of Minimum Number of LCL Cells Required to Give Rise to Subcutaneous Tumours Regularly in SCID Mice

To establish the minimum number of LCL cells required for consistent tumour outgrowth in SCID mice 10^6, $2x10^6$ and $4x10^6$ cells from each LCL were injected subcutaneously into 3 separate SCID mice. Each cell dose was injected into two mice on either side of the body, lateral to the lumbar spine. Animals were observed for 16-20 weeks for gross tumour outgrowth.

2.8.3 *In vivo* Prevention of LCL-induced Subcutaneous Tumours in SCID Mice by *in vitro* Generated Effector Cells.

PBMCs, polyclonal CTLs and peptide-specific CTLs from EBV positive donors were mixed with tumourigenic dose of autologous LCL cells in effector:LCL ratios of 2:1, 1:1, 0.5:1, 0.25:1 and 0.12:1 (4 x 10^6, 2 x 10^6, 10^6, 5 x 10^5 and 10^5 PBMC were mixed with 2 x 10^6 autologous LCL cells). Each mixture was injected subcutaneously in two SCID mice at 2 sites/mouse or as a single midline injection. LCL and effector only controls were included for all experiments. LCL only control received 2 x 10^6 cells and effector only controls received cell numbers equal to the highest dose injected with effector and LCL mixed injections. Animals were observed for gross tumour outgrowth for a 100 days.

2.8.4 *In vivo* Treatment of LCL induced Subcutaneous Tumours in SCID Mice by *in vitro*-generated Effector Cells.

Single midline subcutaneous LCL tumours were induced in SCID mice . Single and multiple fixed dose CTL injections (3 x 10^6 cells) were delivered after appearance of gross tumours. Two routes of CTL delivery intravenous (I/V) and intratumour (I/T) were used. The duration of the experiment was 100 days. Control groups included SCID mice injected with LCL only.

2.9 In situ Hybridization and Immunochemistry

2.9.1 General Methods

2.9.1.1 Silane Coating of Slides

A 2% v/v solution of silane was made in acetone. Slides were immersed in this solution for 20 seconds, followed by a quick rinse in acetone followed by distilled water. Slides were wrapped in foil to protect from dust and dried at 37^{0}C.

2.9.1.2 Cytospin Preparation

10^6 cells were washed twice in PBS and resuspended in 1 ml. 100uls were placed in each well of cytocentrifuge and spun at 150,000 rpm for 3 minutes onto silane

coated glass slides. Slides were air dried and fixed for ten minutes at 4^0C in methanol and/or 4% formalin. Slides were stored at -20^0C.

2.9.1.3 Counterstaining of Slides

Counterstaining was not routinely used with in situ hybridization or immunochemistry techniques because of the possibility of masking a positive signal by the stain. However, in some cases Evan's blue, Neutral Red, Vector Red and Haematoxylin were used.

2.9.1.4 Permanent Mounting of Stained Sections

To avoid the risk of loss of precipitated substrates indicating positive signal by dissolving in non-aqueous mounting media, sections were permanently mounted in Faramount mounting medium (Dako).

2.9.1.5 Haematoxylin and Eosin Staining of Sections

Sections were incubated in Mayer's Haematoxylin (Sigma) for 5 minutes at room temperature then washed and incubated with Eosin for 15 minutes. Slides were washed in tap water and mounted.

2.9.2 In situ Hybridization for Human Cytokine mRNA.

2.9.2.1 Dewaxing & Rehydration

Paraffin was removed from wax embedded sections by two five-minute immersions in Xylene at room temperature. Sections were rehydrated at room temperature in 99% and 95% ethanol twice for one minute. This was followed by a 5 minute wash in DEPC treated water. Signal to noise ratio was enhanced by incubating slides in 2 x SSC at 60^0C for ten minutes.

2.9.2.2 Titration of Proteinase K (PK) Digestion Time and Concentration

For unmasking mRNA signal, sections were treated with PK at 37^0C in a humid chamber. To find a PK concentration which gave optimum tissue digestion for mRNA detection, varying PK concentrations (20ug/ml, 10ug/ml, 8ug/ml and 5ug/ml) were tested for a fixed time period of 30 minutes. All concentrations

resulted in over digestion of section, visible as tissue detachment from slides, over digested vacuolated areas and intensely staining nucleoli. In the next step duration of digestion was titrated for all the concentrations of PK tested earlier. Digestion times tested for each concentration were 20, 15 and 10 minutes. Optimum tissue digestion with maximal preservation of tissue morphology and mRNA signal was achieved with a PK concentration of 10ug/ml for 10 minutes. This concentration was used for paraffin embedded sections in the study. Optimum PK concentration and digestion time for PBMC cytospins was titrated separately. PK concentrations of 5ug/ml, 4ug/ml, 2ug/ml, 1ug/ml and no PK digestion were titrated for 10 and 5 minutes. Optimum signal was achieved with 2ug/ml of PK for 10 minutes.

2.9.2.3 RNase Treatment of Sections

RNase was dissolved in RNase buffer to achieve a concentration of 25 mg/ml and was stored at -20^0C. Working solution was prepared by diluting RNase stock solution in RNase buffer to get a final concentration of 25 ug/ml. Slides for RNase digestion were placed into the RNase solution in a coplin jar, and incubated at 37^0C for 30 minutes. Separate glassware and disposable plastic pipettes were used for the RNase incubation to avoid contamination of non-RNase treated sections.

2.9.2.4 Fixation

PK and RNase digestion was halted by immersing slides in pre-cooled 0.4% w/v solution of paraformaldehyde in 1 x PBS for 20 minutes at 4^0C. Sections were removed and washed once in 1 x PBS for three minutes.

2.9.2.5 Pre-Hybridization and Hybridization

Sections were incubated in 60uls of hybridization buffer for 30 minutes at 37^0C. This was followed by incubation of each section with 20ng of probe for 18-24 hours at 37^0C in a humid chamber. Higher probe concentrations (30-40ng/section) were used in a few runs during standardisation of the technique but did not improve signal detection and were not used routinely.

2.9.2.6 Stringency washes and blocking

To block non-specific signal sections were washed twice for 5 minutes in 4x and 2x SSC at 37^0C and incubated for 15 minutes at R/T in TBS supplemented with 1% BSA, 0.1% Triton X 100 and 0.2mM $MgCl_2$. Sections were further incubated in 2-20% sheep, rabbit and donkey serum at room temperature, for ten minutes each to further block non-specific binding of primary, secondary and tertiary antibodies.

2.9.2.7 Signal detection and amplification

Three protocols were followed to detect cytokine mRNA signal. In protocol 1 sections were incubated for two hours at R/T in a 1:100 dilution of an anti-digoxigenin antibody coupled with alkaline phosphatase (AP). This was followed by an O/N incubation in 5-bromo-4-choloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate. This protocol failed to detect mRNA signal in all tissue tested.

In protocol 2 an indirect approach was taken by using an unlabelled sheep anti-DIG antibody. Sections were incubated O/N at 4^0C in a 1:25-50 dilution of the anti-DIG antibody, followed by a 30 minute incubation at R/T in 1:100 dilution of an anti-sheep antibody labeled with biotin. Signal was detected by incubation of slides in a 1:5000 dilution of streptavidin (Dako) at R/T for 30 minutes followed by a 30 minute incubation at R/T in a 1:1000 dilution of biotin labeled alkaline phophatase (Dako). Substrate used was BCIP/NBT. mRNA signal detected by this method was not strong enough for adequate interpretation.

To further enhance the signal, in protocol 3 extra steps were introduced by including a FITC labeled antibody followed by an anti-FITC antibody. Sections were incubated O/N at 4^0C in a 1:50 dilution of a non-labeled sheep anti-DIG antibody, followed by incubation for 30 minutes at R/T in a 1:100 dilution of a FITC labeled donkey anti-sheep antibody. Slides were further incubated overnight at 4^0C in a 1:50 dilution of a rabbit anti-FITC antibody, followed by incubation for 30 minutes at R/T in a 1:200 dilution of a goat anti-rabbit biotin labeled

antibody. Detection was by the same method as in protocol 2. This protocol gave interpretable results. Table 2.4 shows the steps in the three protocols.

Table 2.4

Three Signal Detection And Amplification Protocols Followed After Incubation Of Sections With Digoxigenin Labeled Oligonucleotide Probes.

1st Protocol	2nd Protocol	3rd Protocol
1. 2 h R/T 1:500 sheep anti-DIG-**AP**	1. O/N 4°C 1:50 sheep anti-DIG.	1. O/N 4°C 1:50 sheep anti-DIG
2. Wash	2. Wash	2. Wash
⇓	**3. 30 min R/T 1:100 rabbit anti-sheep Biotin labeled**	**3. 30 min R/T 1:100 donkey anti-sheep FITC labeled**
	4. Wash	**4. Wash**
	5. 30 min R/T 1:5000 Streptavidin	**5. O/N 4°C 1:50 rabbit anti-FITC**
	6. Wash	6. Wash
	7. 30 min R/T 1:1000 AP-Biotin .	7. 30 min R/T 1:100 goat anti-rabbit Biotin labeled
	8. Wash	8. Wash
	⇓	9. 30 min R/T 1:5000 Streptavidin
		10. Wash
		11. 30 min R/T 1:1000 AP-Biotin
		12. Wash
		⇓
O/N R/T 100ul of ready to use BCIP/NBT (Sigma)		
Sections washed as before and mounted in Faramount mounting medium (Dako)		

(*R/T* room temperature, *O/N* overnight, *Dig* digoxigenin, *TBS* tris buffered saline, *AP* alkaline phosphatase, *BCIP/NBT* 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium, *Wash* 2 x 5 min in 1 X TBS at R/T).

Extra steps added to each protocol are shown in red.

2.9.2.8 Titration of antibody, incubation period and incubation temperature

The anti-DIG and anti-FITC antibodies used in the study (table 2.2) were tested at varying concentration under different incubation conditions for optimal signal detection. Optimum results were obtained at antibody concentrations higher than 1:50 with overnight incubation at 4°C. Shorter incubation time did not give a strong signal and increasing temperature to R/T or 37°C resulted in non-specific signal. Secondary and tertiary antibodies (listed in table 2.3) were used at 1:100 to

1:1000 dilutions at R/T for 30 minutes. Increasing temperature resulted in non-specific signal. Increasing time from 30 minutes to one hour slightly improved signal, but further increase in time caused non-specific binding.

2.9.2.9 Incubation with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT)

BCIP/NBT was the AP substrate of choice for in situ hybridization. This substrate produced a purple/blue precipitate. It gave a good localization of signal and had a high sensitivity. It was also very stable and resistant to alcohol. Therefore sections could be dehydrated and permanently mounted without loss of signal.

2.9.3 In situ Hybridization for EBER Detection

EBER mRNA was detected by using a commercial kit (Hybaid). Manufacturer's instructions were followed with modifications. Sections were dewaxed, rehydrated and PK digested as described above. PK digestion was halted by a quick rinse in PBS, followed by dehydration of sections in 95% and 99% ethanols respectively (2 x 1 minute in each). Sections were air dried and incubated with one drop of pre-diluted FITC labeled probe at 60^0C for ten minutes and then at 37^0C for two hours. Slides were washed three times in 1 x TBS and incubated with a drop of a secondary AP conjugated anti-FITC antibody for two hours at room temperature. Signal was detected by substrate (BCIP/NBT) precipitation. Sections were mounted in Faramount mounting medium.

2.9.4 Immunochemistry for Detection of Cell Surface Markers

Microwaving of Sections

Microwaving of sections was used for immunophenotyping of tumour sections. Wax embedded sections were dewaxed and rehydrated as described for NISH. Slides were microwaved three times for five minutes at 850 watts in citrate buffer to expose cell surface antigens. To prevent sections from drying out during the procedure evaporated solution was replaced with DEPC treated water between each five minute treatment. Cytospin preparations were not microwaved.

Immunochemistry

After microwaving slides were allowed to cool in the citrate buffer to R/T and washed twice in PBS. Endogenous peroxidase was blocked by incubating sections in 0.3% hydrogen peroxide for ten minutes if HRP was used as the detection system. Sections were incubated with primary antibody O/N at either R/T or 4^0C at the concentration recommended by the manufacturer. Slides were washed and incubated in a biotin labeled secondary antibody at a concentration recommended by the manufacturer. Signal was detected using ABC-AP or ABC-HRP kits (Dako) following manufacturer's instructions. Substrate used for HRP was DAB and for AP was BCIP/NBT. Sections were permanently mounted in Farmount mounting medium (Dako).

Microscopy

Signal was seen using standard bright field transillumination. Final magnifications of 100 x, 200 x and 400 x were used to detect hybridisation signal (visible as purple-blue deposits over cells expressing the target mRNA) and to calculate the percentage of positive cells in relation to the total number of cells positive for β-actin mRNA.

3. RESULTS 1

3.1 Pre-Clinical Tumour Model For Human EBV Positive Post Transplant Lymphoproliferative Disease

This study was designed to test autologous unfractionated PBMC, polyclonal EBV-specific and peptide specific cytotoxic T lymphocytes (CTL) from EBV seropositive donors in prevention and treatment of EBV positive subcutaneous human B cell tumours in a pre-clinical model, using SCID mice.

3.1.1 *In vitro* Generation and Testing of LCL from Ten Healthy EBV Seropositive Blood Donors

To standardize an EBV positive human B cell tumour model in the SCID mouse, a panel of ten human LCLs (No. 1-10) were grown *in vitro* by infection of PBMC from EBV seropositive donors with concentrated EBV preparation. EBV status of 5 LCLs was confirmed by staining cytospin preparations for expression of the EBNA complex in an anti-EBNA immunoflourescence assay (kindly carried out by Miss Kate Britton, Medical Microbiology, University of Edinburgh). All five LCLs were positive for expression of EBNA complex. Cytospin preparations from five LCLs were stained for CD20, a B cell marker. Over 99% cells were positive for expression of CD20, confirming the B cell lineage of the LCLs.

3.1.2 Standardization of a Subcutaneous EBV Positive B Cell Tumour Model in SCID Mice

A subcutaneous EBV positive tumour model was designed to study the effect of autologous PBMC, polyclonal EBV-specific CTLs and peptide-specific CTLs on LCL tumour outgrowth in the SCID model.

3.1.3 Establishment of Minimum Number of LCL Cells Required to Consistently Give Rise to Subcutaneous Tumours in SCID Mice

The minimum LCL cell number required for consistent tumour outgrowth in SCID mice was established by subcutaneous injections of 10^6, 2×10^6 and 4×10^6 cells from each LCL into 3 separate SCID mice at 2 sites per mouse. Overall all cell numbers injected resulted in subcutaneous tumours. Doses of 2×10^6 cells and

above resulted in tumour occurrence in 100% cases and average time to appearance of gross tumour was 5.5 weeks for $2x10^6$ and 5 weeks for $4 x10^6$ cells. The incidence of tumour generation with 10^6 cells was 55% and average time to tumour outgrowth was 10 weeks. It was concluded that a 2×10^6 was the minimum cell number required for regular tumour generation and this cell number was used as the standard dose for subcutaneous tumour induction in SCID mice. Results of the experiment are shown in table 3.1. Details of time to tumour development for individual donors is listed in table 3.2

TABLE 3.1

Percentage of tumours seen with different LCL cell numbers injected subcutaneously in SCID mice.

Number of LCL Cells Injected	Percent Tumours Outgrowth	Average time to tumour in weeks
10^6	55%	10
2×10^6	100%	5.5
4×10^6	100%	5

TABLE 3.2

Time in Weeks To Tumour Development for Individual Donor LCLs

DONOR	LCL CELL NUMBER INJECTED		
	10^6	2×10^6	4×10^6
	SITE 1 / 2	SITE 1 / 2	SITE 1 / 2
1	10 / 09	04 / 07	3.5 / 05
2	NT / NT	05 / 05	04 / 06
3	07 / NT	07 / 06	07 / 07
4	09 / NT	04 / 05	04 / 06
5	11 / NT	4.5 / 06	4.5 / 05
6	15 / NT	4.5 / 06	04 / 06
7	8.5 / 10	04 / 05	3.5 / 4.5
8	12 / 13	05 / 6.5	4.5 / 05
9	NT / NT	07 / 08	05 / 06
10	09 / NT	4.5 / 07	04 / 04

NT: No Tumour

3.2 Immunotherapy Of EBV Positive Subcutaneous Tumours in the SCID Mouse Model

3.2.1 Effect of Unfractionated PBMCs on Outgrowth of LCL Induced Subcutaneous Tumours In SCID Mice

Initially the effect of unfractionated autologous PBMCs from EBV seropositive donors was assessed on the outgrowth of EBV positive tumours in SCID mice. This was done to establish if T cells from within the PBMC population would inhibit tumour outgrowth in the SCID mice. PBMC from three donors were mixed with autologous LCL cells in PBMC:LCL ratios of 2:1, 1:1, 0.5:1, 0.25:1 and 0.12:1. Each mixture was injected subcutaneously at a single midline site in two SCID mice each. Mice engrafted with LCL ($2x10^6$ cells) and PBMC (4×10^6) only were included as controls for all three donors. Tumours developed in all the LCL only controls within 5-7 weeks whereas PBMC on their own did not cause tumours in any case. PBMC from all three donors failed to alter tumour outgrowth at PBMC:LCL ratios of 0.5:1 and below. PBMC from donor 3 prevented one tumour at the PBMC:LCL ratio of 1:1. However, the same donor's PBMCs failed to affect the LCL tumour outgrowth at the higher PBMC:LCL ratio of 2:1. PBMC from donor 4 prevented half of the tumours at PBMC:LCL ratio of 1:1 and 2:1. PBMC from donor 7 did not affect tumour outgrowth at any PBMC:LCL ratio. Since the number of animals in each group was small, statistical significance of the results could not be measured. Results are shown in table 3.3

Table: 3.3

Effect of by Autologous Unfractionated PBMC on Outgrowth of LCL induced tumours in SCID mice

PBMC:LCL RATIOS INJECTED										
Donor	**2:1**		**1:1**		**0.5:1**		**0.25:1**		**0.12:1**	
	1	2	1	2	1	2	1	2	1	2
3	+	+	-	+	+	+	+	+	+	+
4	-	+	-	+	+	+	+	+	+	+
7	+	+	NT	+	NT	+	+	+	+	+

+ = Gross subcutaneous tumour, - = No Tumour, NT = Not Tested

3.2.2 Effect of Autologous Polyclonal EBV-Specific CTLs on Outgrowth of LCL Induced Subcutaneous Tumours In SCID Mice

The next set of experiments was designed to test if *in vitro* expanded EBV-specific CTLs would affect the outgrowth of autologous LCL tumours *in vivo*.

3.2.2.1 *In vitro* Generation and Testing of EBV Specific Polyclonal CTLs

EBV-specific polyclonal CTLs were generated from PBMC of ten donors and expanded *in vitro* for a minimum of 4-6 weeks. The immunophenotype of all ten CTL lines was determined by flow cytometry (kindly done by Mr. Alan Ross, Veterinary Pathology, University of Edinburgh). Overall percentage of CD3 positive cells ranged between 72-97%. Percentage of CD8 positive T cells ranged between 28-67%. CD4 positive cells ranged between 8-43%. CTLs were also tested for their cytotoxic activity against autologous and allogeneic LCLs in a standard four hour ^{51}Cr release assay.

3.2.2.2 Results of Standard 4 Hour ^{51}Cr release assay to Test Cytotoxic Activity of CTLs

Specific lysis of autologous LCL and allogeneic LCL cells by each CTL was tested in a standard four hour ^{51}Cr release assay. NK cell activity (non-HLA restricted killing) was assessed by including the K562 cell line as a target. All ten CTLs showed higher killing of autologous LCLs as compared to the HLA mismatched LCL and K562 cells. Results are displayed in figure 3.1. Overall lysis of LCLs by autologous CTL lines varied from 35% to 70%. Lysis of allogeneic LCLs and K562 cells ranged from -8% to 20% and -20% to 28% respectively. Data from all ten donors was pooled and statistical analysis was carried out using Chi-square test. Results showed a significant difference between percent specific lysis of autologous and HLA mismatched LCLs ($p<0.001$).

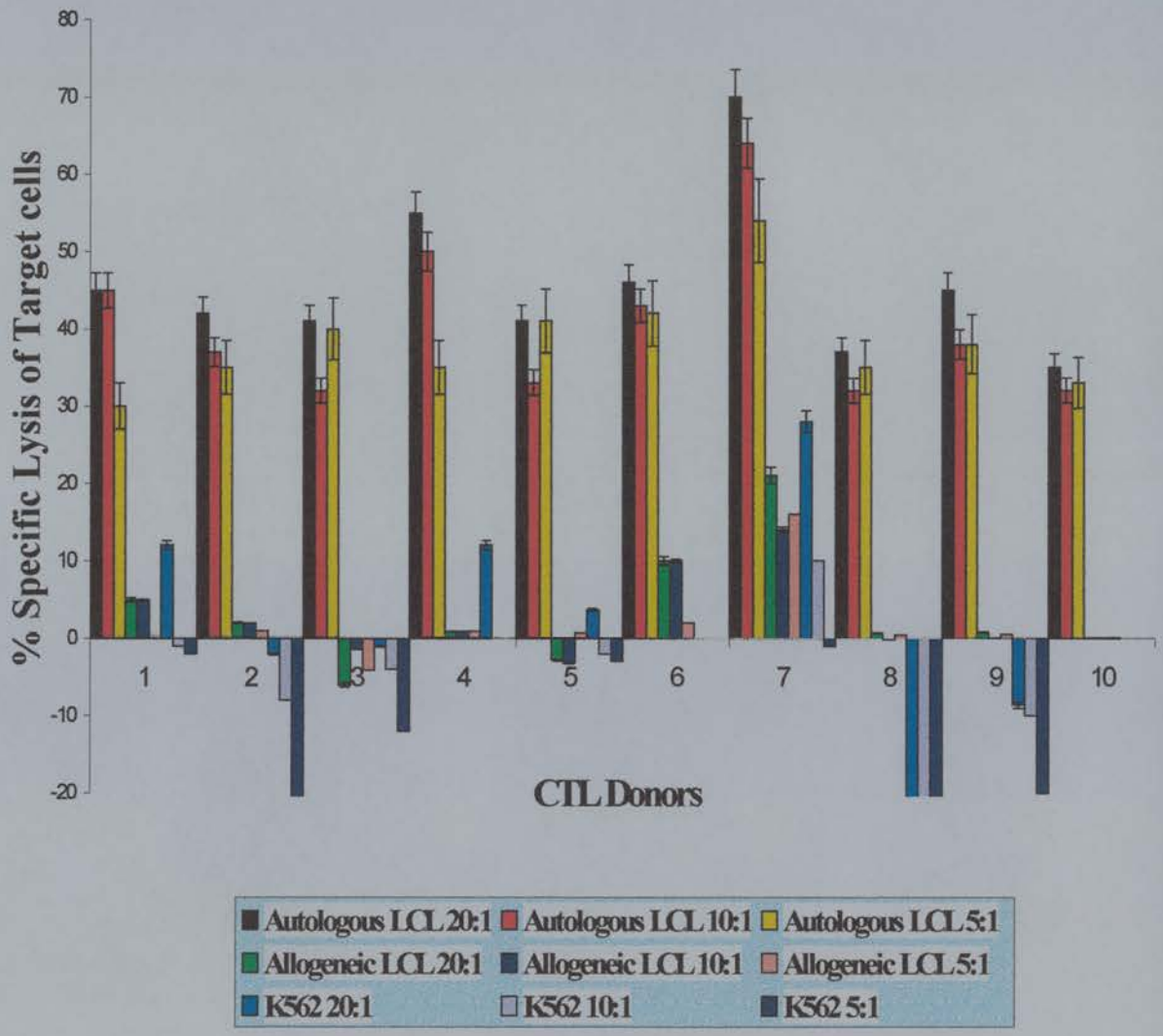
3.2.2.3 *In vivo* Effect of Autologous CTL on Outgrowth of Subcutaneous Autologous LCL Tumours in SCID Mice

To observe if *in vitro* generated EBV-specific CTLs would alter outgrowth of autologous LCL tumours in SCID mice CTL cells were mixed with autologous LCL cells in ratios 2:1, 1:1, 0.5:1, 0.25:1, 0.12:1, 0.06:1 and 0.03:1 and each

mixture was injected subcutaneously in 2 SCID mice at 2 sites/mouse. Separate animals injected with LCL cells ($2x10^6$) and CTL cells ($4x10^6$) alone were included as controls for all ten donors.

Figure 3.1

Lysis of Autologous and Allogeneic Targets by CTLs in a Standard ^{51}Chromium Release Assay



The animals were observed regularly for tumour outgrowth for a period of 100 days and time to tumour development was also noted. All animals in the LCL only control group developed tumours within 6 weeks. None of the mice in CTL only control group developed tumours. Tumour incidence was zero for animals

injected with CTL:LCL ratios: ≥ 0.5:1. 30% animals developed tumours at CTL:LCL ratio of 0.25:1 and tumour incidence was 100% in animals injected with CTL:LCL ratios below 0.25:1. Results are shown in table 3.5. Table 3.6 shows the time to tumour development in weeks for different groups of animals. The average time to tumour in the LCL only control group was 5.5 weeks, while the average time to tumour outgrowth was 7.1 weeks for animals which received lower CTL and LCL ratios and developed tumours. This delay in tumour outgrowth was also observed in the group which received the lowest CTL number (CTL:LCL ratio of 0.03:1). Results from each CTL:LCL ratio from all the donors were pooled together and statistical analysis was performed using Chi-Square test with application of Yate's correction. Analysis showed that, compared to LCL only control group autologous CTL significantly prevented tumour outgrowth down to CTL: LCL ratio 0.25:1 ($P < 0.01$).

TABLE 3.5

Outgrowth Of EBV Positive Subcutaneous Tumours In SCID Mice Receiving Varying Concentrations of EBV Specific Cytotoxic T-Cells.

	CTL	LCL	CTL:LCL RATIOS INJECTED						
	$4X10^6$	**$2X10^6$**	**2:1**	**1:1**	**0.5:1**	**0.25:1**	**0.12:1**	**0.06:1**	**0.03:1**
DONOR			SCID mouse 1 / mouse 2						
			1 / 2	1 / 2	1 / 2	1 / 2	1 / 2	1 / 2	1 / 2
1	-	+	-	-	-	+ / -	+	+	+
2	-	+	-	-	-	-	+	+	+
3	-	+	-	-	-	-	+	+	+
4	-	+	-	-	-	-	+	+	+
5	-	+	-	-	-	- / +			NT
6	-	+	-	-	-	+ / -	NT	NT	NT
7	-	+	-	-	-	-	+	+	+
8	-	+	-	-	-	+ / -		NT	NT
9	-	+	-	-	-	- / +	NT	NT	NT
10	-	+	-	-	-	- / +	NT	NT	NT

+ = Positive For Gross Tumour, - = No Gross Tumour, NT = Not Tested

Table 3.6

Time To Tumour Development (In Weeks) following injection of LCL, CTL and CTL:LCL mixture in SCID mice.

	CTL	LCL	CTL:LCL RATIOS						
	4 x 10^6 Cells	**2 x 10^6 Cells**	**2:1**	**1:1**	**0.5:1**	**0.25:1**	**0.12:1**	**0.06:1**	**0.03:1**
DONOR			**SCID Mouse 1 / Mouse 2**						
			1 / 2	1 / 2	1 / 2	1 / 2	1 / 2	1 / 2	1 / 2
1	-	4	-	-	-	7 / -	9	6	7
2	-	5	-	-	-	-	7	7	6
3	-	5	-	-	-	-	8	9	8
4	-	6	-	-	-	-	6	6	7
5	-	5	-	-	-	- / 6			
6	-	7	-	-	-	8 / -	NT	NT	NT
7	-	6	-	-	-	-	7	7.5	6
8	-	4	-	-	-	7 / -	NT	NT	NT
9	-	7	-	-	-	- / 8	NT	NT	NT
10	-	6	-	-	-	- / 7	NT	NT	NT

- = No Tumour, NT = Not tested

3.2.3 Effect of Autologous EBV Lytic and Latent Antigen-Specific CTL on Outgrowth of Subcutaneous Autologous LCL Tumours

Since polyclonal CTLs from all ten donors altered the growth of autologous LCL tumours at very low CTL:LCL ratios, in the next step we observed the effect of EBV peptide-specific CTLs on outgrowth of autologous LCL tumours.

3.2.3.1 *In vitro* Generation of Peptide Specific CTLs

Peptide-specific CTLs were generated *in vitro* from donor PBMC using a panel of ten, 8-10mer long peptides. Autologous peptide pulsed PBMC were used as stimulators.

Individual aliquots of PBMC were pulsed with exogenous peptides representing immunodominant epitopes from EBV lytic cycle antigens gp350 and BMLF1 and latent cycle antigens: EBNA 3, 4, 6 and LMP1. EBV antigens represented by the peptides used, peptide sequences, their position in the primary antigen sequence and HLA restriction are listed in table 3.7. The donors and their HLA type are shown in table 3.8.

Table 3.7

Peptides Representing Known Immunodominant Epitopes from within Primary Sequences of EBV Lytic and Latent Cycle Antigens

EBV Protein	Residue	Epitope Sequence	HLA Restriction
LYTIC CYCLE ANTIGENS			
BMLF1	280-288	GLCTLVAML	A2
gp350	863-871	VLQWASLAV	A2
LATENT CYCLE ANTIGENS			
EBNA3	491-499	VFSDGRVAC	A29
EBNA3	596-604	SVRDRLARL	A2
EBNA3	458-466	YPLHEQHGM	B35
EBNA4	657-666	VIETPYKPTW	B44
EBNA6	335-343	KEHVIQNAF	B44
EBNA6	281-290	EFNLLDFVRF	B44
LMP1	125-133	YLLEMLWRL	A2

Adapted from Rickinson & Moss, 1996

Table 3.8

HLA Types of Donors Selected for Generation of HLA restricted EBV Peptide-Specific CTL lines.

DONOR	HLA TYPE			
	HLA A		**HLA B**	
2	2	19	12	44
5	2	29	11	44
6	2	11	15	35
7	1	2	8	44

3.2.4 Analysis of CTL Response to Peptides Representing Immunodominant Epitopes from EBV Lytic and Latent Cycle Antigens in Donor 5.

A panel of six peptide-specific CTLs were generated from PBMC of donor 5. The peptides used and their properties are listed in table 3.9.

Table 3.9

Peptides used to Expand EBV epitope specific CTL lines from donor 5. Sequences and HLA restriction of each peptide are listed.

No	EBV Protein	Residue	Epitope Sequence	HLA Restriction
LYTIC CYCLE ANTIGENS				
1	BMLF1	280-288	GLCTLVAML	A2
2	gp350	863-871	VLQWASLAV	A2
LATENT CYCLE ANTIGENS				
3	EBNA3	491-499	VFSDGRVAC	A29
4	EBNA4	657-666	VIETPYKPTW	B44
5	EBNA6	335-343	KEHVIQNAF	B44
6	EBNA6	281-290	EFNLLDFVRF	B44

3.2.4.1 Determination of Frequency of EBV Epitope Specific CTL in Peripheral Blood of Donor 5

In order to establish if there was a relationship between precursor frequency of a particular peptide-specific CTL with its ability to prevent LCL induced tumours *in vivo*, the frequency of each ep itope-specific CTL was determined in donor 5 PBMC. CTLp were non-detectable for BMLF1, gp350, EBNA3 and EBNA4 epitopes in donor 5 PBMC. However, CTLp for the two epitopes from within EBNA6 were detected. Precursor frequency for KEHVIQNAF was 40 per 10^6 cells and that for EENLLDFVRF was 11 per 10^6 PBMC. Results are shown in table 3.10 and figure 3.2.

Table 3.10

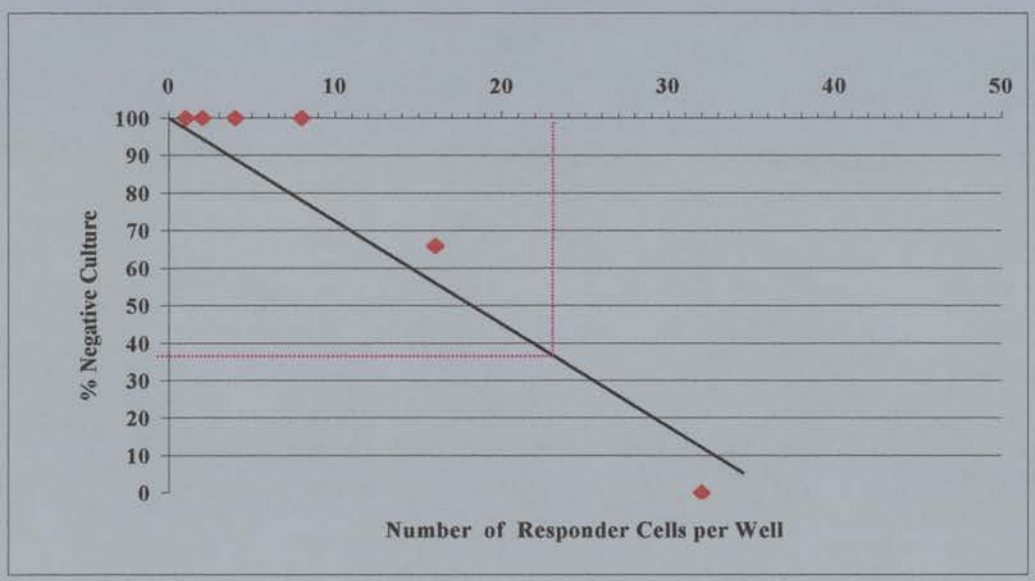
EBV Epitope-Specific CTL Precursor Frequency in Peripheral Blood of Donor 5

EBV Protein	Epitope Sequence	HLA Restriction	CTLp Frequency per 10^6 PBMC
LYTIC CYCLE ANTIGENS			
BMLF1	GLCTLVAML	A2	Undetectable
GP350	VLQWASLAV	A2	undetectable
LATENT CYCLE ANTIGENS			
EBNA3	VFSDGRVAC	A29	Undetectable
EBNA4	VIETPYKPTW	B44	Undetectable
EBNA6	KEHVIQNAF	B44	40
EBNA6	EENLLDFVRF	B44	11

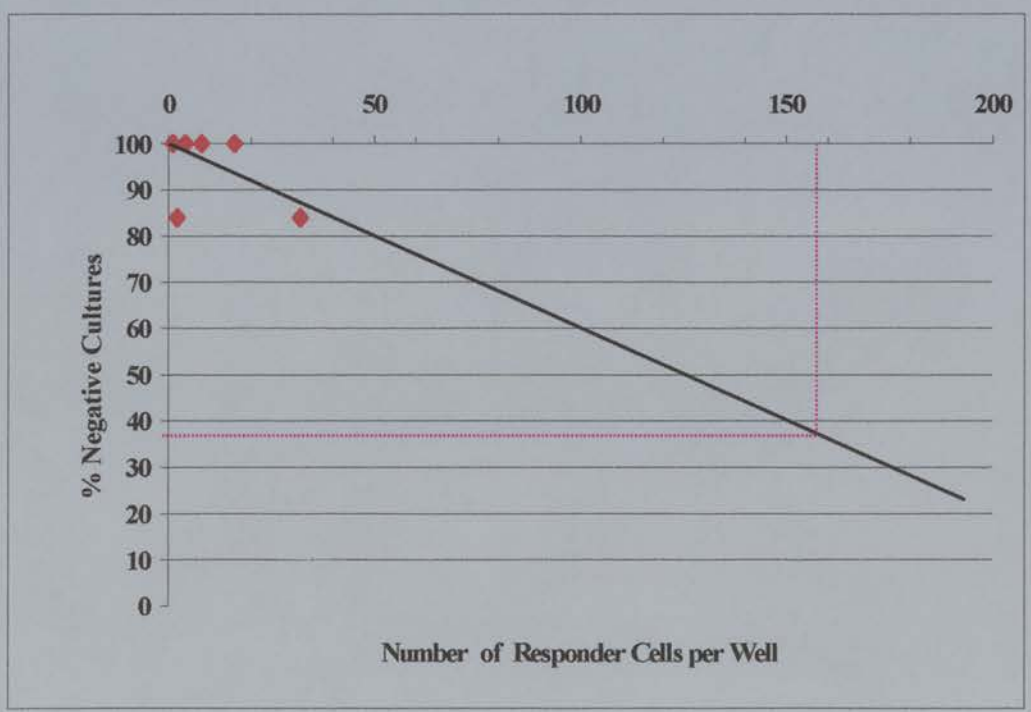
Figure 3.2

Poisson Distribution Slope of Relationship Between Number of Responding and Percentage of Non-Responding Wells in LDA for Peptide-Specific CTLp in Donor 5 PBMC.

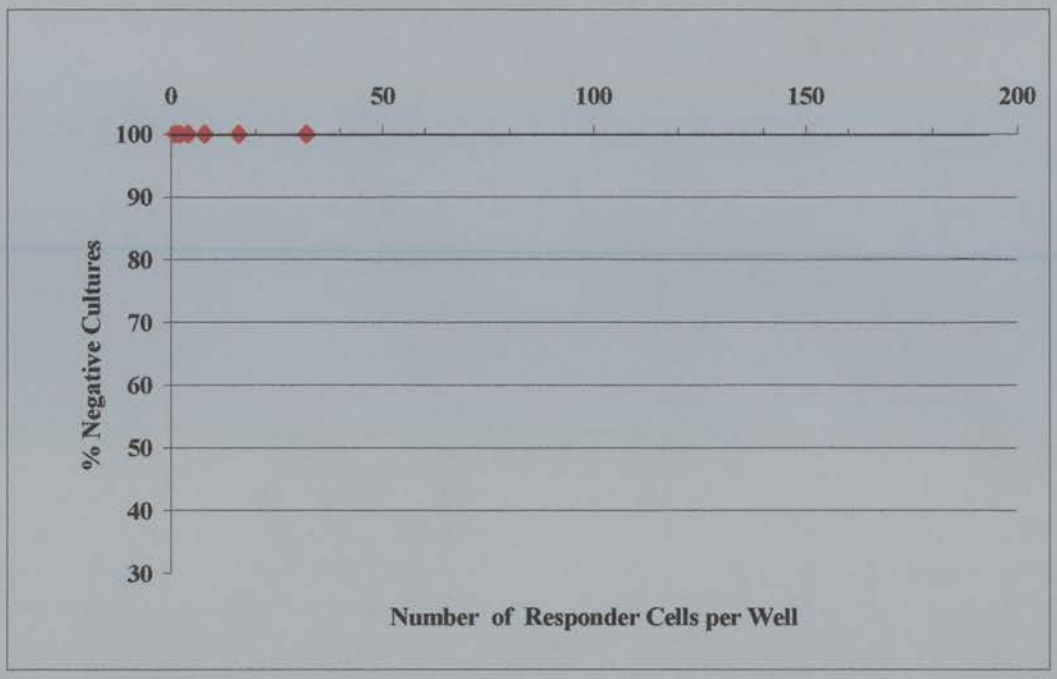
a, Frequency of KEHVIQNAF Specific CTLp



b, Frequency of EENLLDFVRF Specific CTLp



c, Undetectable CTLp for EBNA3



3.2.4.2 ^{51}Cr release assay on Peptide-Specific CTLs from Donor 5

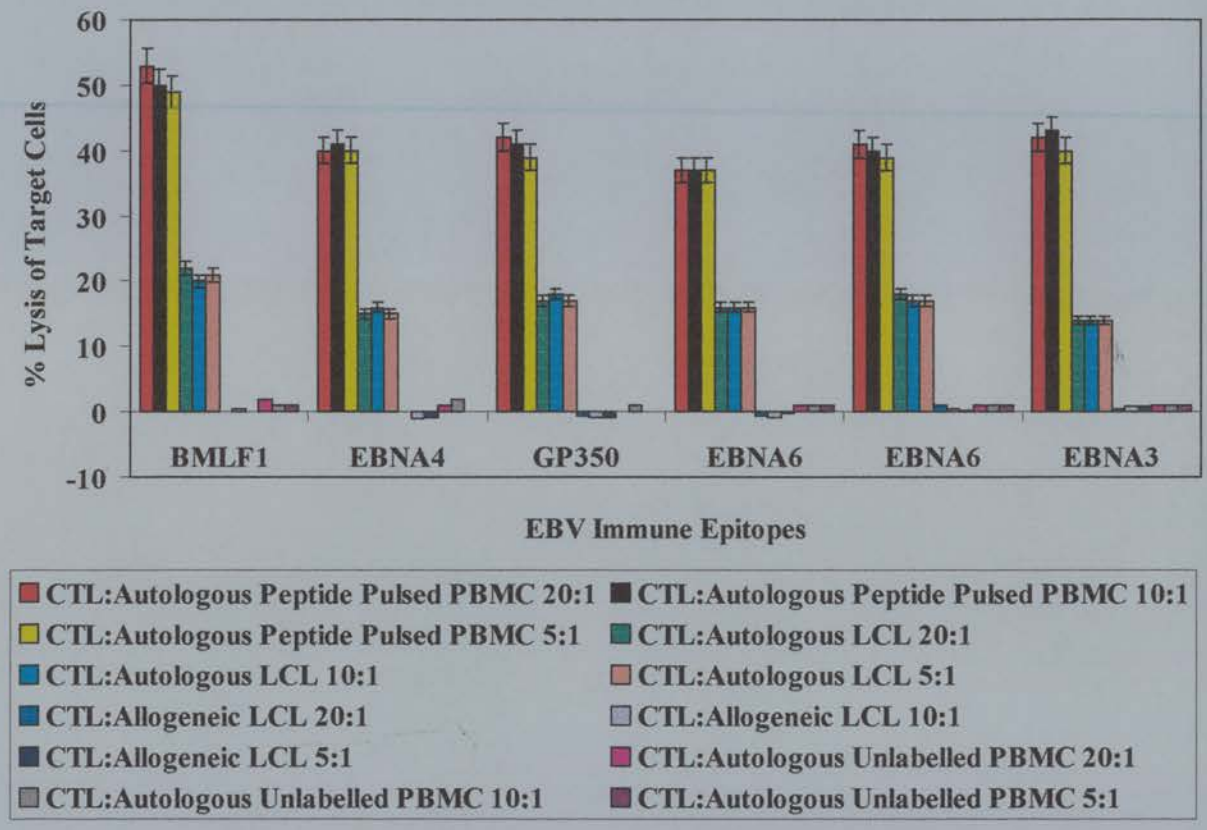
The specificity of each peptide-specific CTL was tested in a standard four hour ^{51}Cr release assay. Targets included autologous PBMC coated with stimulator peptides, autologous LCL cells, unlabeled PBMC and allogeneic LCL cells. *In vitro* specific lysis of autologous peptide pulsed PBMCs by all CTLs varied between 37-53%. The highest percent lysis (53%) was shown by CTL directed against the epitope from the EBV lytic cycle antigen BMLF1, and the lowest (37%) with CTL line directed against the KEHVIQNAF epitope of EBNA6. Lysis of autologous LCL varied between 14-22%. Highest percent lysis (22%) was observed with CTL specific for BMLF1 and lowest (14%) with CTL specific for the EBNA3 epitope. Lysis of allogeneic LCL cells and unlabelled autologous PBMC remained below 5%. Results of the cytotoxicity assay are shown in figure 3.3.

3.2.4.3 *In vivo* Testing of Peptide-Specific CTLs From Donor 5 In Prevention of Autologous LCL Tumours in SCID Mice

Each peptide-specific CTL was mixed with $2x10^6$ autologous LCL cells at CTL:LCL ratios of 1:1, 0.5:1, 0.25:1, 0.12:1 and 0.06:1 and injected as a single

Figure 3.3

Lysis of Autologous and Allogeneic Targets by CTLs in a Standard ^{51}Chromium Release Assay



midline subcutaneous injection in SCID mice. Each ratio was tested in duplicate in two separate animals. Two LCL ($2x10^6$ LCL cells each) and one CTL ($2x10^6$ CTL cells) only controls for each of the six CTLs were included. Tumours developed in both LCL only controls within six weeks. None of the animals injected with CTL only developed tumours. Mice which received LCL cells mixed with CTLs specific for GP350, BMLF1, EBNA3, EBNA4 and EENLLDFVRF epitope from EBNA6 developed tumours at CTL:LCL ratios below 1:1 within 6-10 week. However, in the group which received CTLs specific for the EBNA6 KEHVIQNAF epitope, only one mouse developed a tumour at the lowest CTL:LCL ratio injected (0.06:1). Results are shown in table 3.11.

TABLE 3.11

Tumour Outgrowth In SCID Mice Injected with EBV Peptid-Specific CTL Lines from Donor 5

	Subcutaneous Tumours in SCID Mice									
	CTL:LCL RATIOS INJECTED									
	1:1		0.5:1		0.25:1		0.12:1		0.06:1	
	SCID MICE									
	1	2	1	2	1	2	1	2	1	2
EBV Peptide-Specific CTLs										
BMLF1	-	-	+	+	+	+	+	+	+	+
GP350	+	+	+	+	+	+	+	+	+	+
EBNA3	NT	NT	NT	NT	+	+	+	+	+	+
EBNA6	-	-	-	-	-	-	-	-	-	+
EBNA6	-	-	-	+	+	+	+	+	+	+
EBNA4	-	-	+	+	+	+	+	+	+	+

+ = Gross subcutaneous tumour - = No Tumour NT = Not tested.

All CTL only control animals remained tumour free

Both mice in LCL only control groups developed tumours.

3.2.5 Analysis of CTL Response to Peptides Representing Immunodominant Epitopes from EBNA3 and EBNA6 in Donor2 PBMC

In the next set of experiments peptide-specific CTLs were expanded from donor 2 PBMCs and CTLp frequency for each was measured (the techniques used were same as in section 3.2.3). The HLA type of Donor 2 was HLA A2, A19, B12, B44. PBMC were pulsed with exogenous peptides representing one immunodominant HLA A2 restricted EBNA3 epitope; SVRDRLARL and two HLA B44 restricted EBNA6 epitopes KEHVIQNAF and EENLLDFVRF. CTLp frequency for each epitope was measured. CTLp were detectable for all three epitopes in the PBMC of donor 2. Precursor frequency for CTL directed against the EBNA3 SVRDRLARL epitope was 123 CTLp per 10^6 PBMC, and for the EBNA6 EENLLDFVRF and KEHVIQNAF epitopes was 114 and 48 CTLp per 10^6 PBMC respectively. Results are shown in table 3.12 and the Poisson distribution is shown in figure 3.4.

Table 3.12

EBV Epitope-Specific CTL Precursor Frequency in Peripheral Blood of Donor 2

EBV Protein	Epitope Sequence	HLA Restriction	CTLp Frequency per 10^6 PBMC
EBNA3	SVRDRLARL	A2	123
EBNA6	KEHVIQNAF	B44	48
EBNA6	EENLLDFVRF	B44	114

3.2.5.1 *In vivo* Testing of Peptide-Specific CTLs From Donor 2 In Prevention of Autologous LCL Tumours in SCID Mice

Each peptide-specific CTL was mixed with $2x10^6$ autologous LCL cells at CTL:LCL ratios of 1:1, 0.5:1, 0.25:1, 0.12:1 and 0.06:1 and injected as a single midline subcutaneous injection in SCID mice. Each ratio was tested in duplicate in two separate animals. Two LCL and one CTL only controls for all the three CTLs were included. Tumours developed in LCL only controls within six weeks. CTLs on their own did not cause tumours. Mice which received LCL cells mixed with peptide-specific CTLs developed tumours at CTL:LCL ratios below 0.25:1 and below within 6-10 week. One animal out of the two from each group which received the two EBNA6 epitope-specific CTLs also developed tumours at CTL:LCL ratio of 0.5:1. Results are shown in table 3.13.

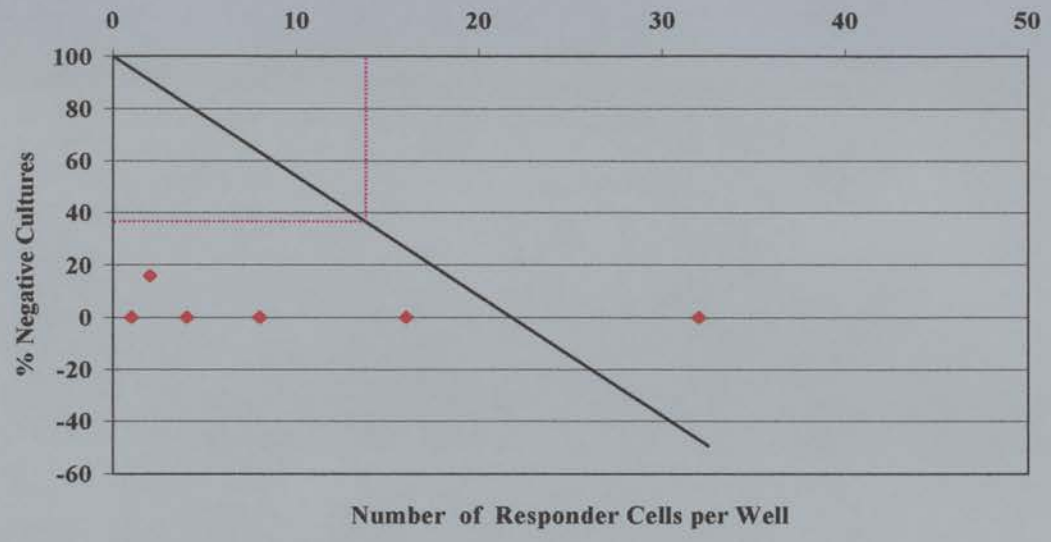
3.2.6. Characterization of *In vivo* Effect of CTL Response to an LMP1 Epitope-Specific CTL on Autologous LCL tumours in SCID Mice.

In the next experiment effect of CTLs targeting a subdominant epitope from LMP1 on outgrowth of autologous LCL tumours was investigated. CTL specific for an HLA A2 restricted LMP1 epitope YLLEMLWRL representing residues 125-133 from within LMP1 sequence were generated *in vitro* from PBMC of Donors 6 and 7. The HLA type of donor 6 was HLA A2, B8, B44 and donor 7 was HLA A2, A11, B8, B35. YLLEMLWRL-specific CTL were mixed with $2x10^6$ LCL cells at CTL:LCL ratios of 1:1, 0.5:1, 0.25:1, 0.12:1 and 0.06:1.

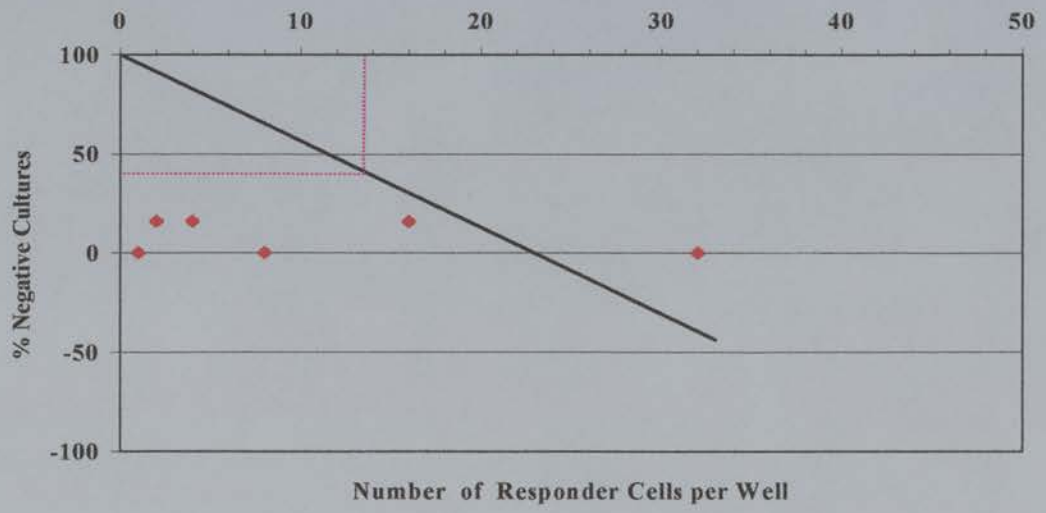
Figure 3.4

Poisson Distribution Slope of Relationship Between Number of Responding and Percentage of Non-Responding Wells in LDA for Peptide-Specific CTLp in Donor 2 PBMC.

a, Frequency of SVRDRLARL Specific CTLp



b, Frequency of EENLLDFVRF Specific CTLp



c, Frequency of KEHVIQNAF Specific CTLp

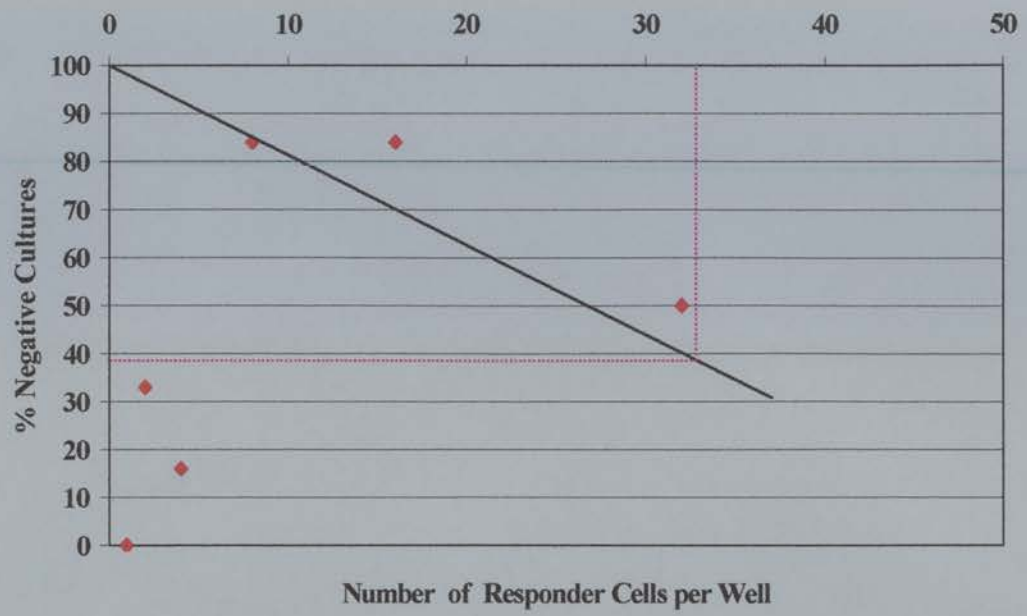


TABLE 3.13

Tumour Outgrowth in SCID Mice Injected with EBV Peptide-Specific CTL Lines from Donor 2

	Subcutaneous Tumours in SCID Mice									
	CTL:LCL RATIOS INJECTED									
	1:1		0.5:1		0.25:1		0.12:1		0.06:1	
	SCID MICE									
	1	2	1	2	1	2	1	2	1	2
EBV Peptide-Specific CTLs										
EBNA3	-	-	-	-	+	+	+	+	+	+
EBNA6	-	-	-	+	+	+	+	+	+	+
EBNA6	-	-	-	+	+	+	+	+	+	+

+ = Gross subcutaneous tumour - = No Tumour NT = Not tested.

All CTL only control animals remained tumour free

Both mice in LCL only control groups developed tumours.

Each ratio was injected in duplicate in two separate animals. CTL and LCL only controls were included. Tumours developed within six weeks in LCL only controls from both donors. No tumours were seen in the CTL only control group. CTLs from donor 6 prevented tumours in CTL:LCL ratios of 0.25:1 and above. Animals injected with the lower CTL:LCL ratio developed tumours at 7 and 8

weeks respectively. In contrast, donor 7 LMP1-specific CTLs failed to control *in vivo* proliferation of autologous LCL cells at all CTL:LCL ratios tested. Tumours occurred in all animals within 8 weeks. Results are shown in Table 3.14.

Table 3.14

***In vivo* autologous LCL induced subcutaneous tumour outgrowth in SCID mice Injected with LMP11 Epitope specific CTLs**

CTL:LCL RATIOS INJECTED					
	1:1	0.5:1	0.25:1	0.12:1	0.06:1
Donor	SCID Mice				
	1 /2	1 / 2	1 / 2	1 / 2	1 / 2
6	- /-	- / -	- / -	+ / +	+ / +
7	+ / +	+ / +	+ / +	+ / +	+ / +

+ = Positive for tumour, - = No gross tumour

3.3 Adoptive Immunotherapy of Subcutaneous EBV Positive Tumours in the SCID Mouse Model

The next step in the study was to test the polyclonal CTLs for their ability to cause regression of already established autologous LCL induced subcutaneous tumours in the SCID model.

3.3.1 Therapy of Established LCL induced Subcutaneous Tumours with a Single Fixed Dose Autologous CTL Infusion

Four donors; 1, 2, 4, 5 were selected from the original panel of ten for an initial pilot study. Single midline subcutaneous LCL tumours were induced in three SCID mice for each donor. In each group one mouse served as a no therapy LCL only control and the other two received autologous CTLs for tumour therapy. I/V and I/T routes of CTL delivery were compared in separate mice for each donor. In preliminary experiments a single fixed dose injection of 3 x 10^6 CTLs was delivered in each case after appearance of gross tumours. The dose of CTL selected was the average of the highest two doses of CTLs ($2x10^6$ and $4x10^6$) used in the previous experiment with polyclonal CTLs, which resulted in 100% inhibition of tumour outgrowth.

3.3.1.1 Measurement and Monitoring of Tumour Mass

Gross tumour mass was measured in cubic millimeters (mm^3) by taking two measurements, 90 degrees to each other using Vernier calipers. Tumour mass was calculated with the formula given below (Conway et al, 2000).

$$\text{Tumour Mass (tm)} = ab^2/2 = \text{(tm) } mm^3$$

Where a = length, b = width

Tumour mass was measured on Day 1 (prior to CTL injection delivery) and then every other day thereafter. The duration of the experiment was 100 days. Reduction in the rate of increase of tumour mass was observed in all treated cases as compared to the rate of increase of tumour mass in the untreated animals (figures 3.5-3.8). This decrease in rate of tumour growth was apparent within 2-3 days of CTL delivery. Apart from two all animals in both I/V and I/T treated groups showed a decrease in the tumour mass after the CTL injection and in 20% of cases a total disappearance of gross tumour was observed.

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3.3.1.2 Response to Intra-venous Adoptive CTL Therapy

Reduction in tumour size with I/V injections was more rapid as compared to the reduction seen with I/T CTL delivery. Overall three out of four (75%) mice treated with I/V CTL showed complete disappearance of gross tumour and were tumour free at the end of the experiment (day 100). The fourth showed an initial decrease in tumour mass but then a rapid increase in tumour size on day 14 (two weeks after injection). The animal was sacrificed once tumour mass increased beyond 10% total body weight.

3.3.1.3 Response to Intra-tumour Adoptive CTL Therapy

Amongst the animals which received I/T CTL injections, all showed reduction in the rate of increase of tumour mass as compared to the non treated control group. Donor 1 and 2 CTLs failed to successfully control tumour growth and animals were sacrificed due to rapid increase in tumour growth rate. Both donor 4 and 5 CTLs caused a complete but transient disappearance of gross tumour but were eventually sacrificed due to rapid increase in tumour mass. Results of the

experiment are displayed in figure 3.5 to 3.8. Survival analysis by Kaplan-Meier method showed that the probability of survival for the non-treated control group and I/T treated group became zero on day 10 and 40 respectively (figure 3.9). However, it remained steady at 0.75 for the I/V treated group, suggesting that I/V delivery of CTL is a better route of delivery for the cells to target the tumour cells via its blood supply.

Figure 3.5

I/T & I/V Treatment of Subcutaneous LCL induced tumours in SCID mice with autologous polyclonal CTL from donor 1.

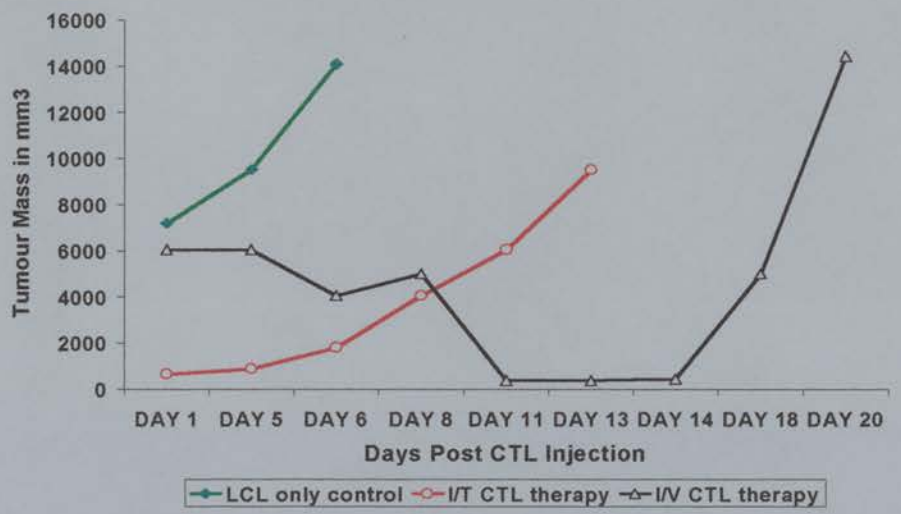


Figure 3.6

I/T & I/V Treatment of Subcutaneous LCL induced tumours in SCID mice with autologous polyclonal CTL in donor 2.

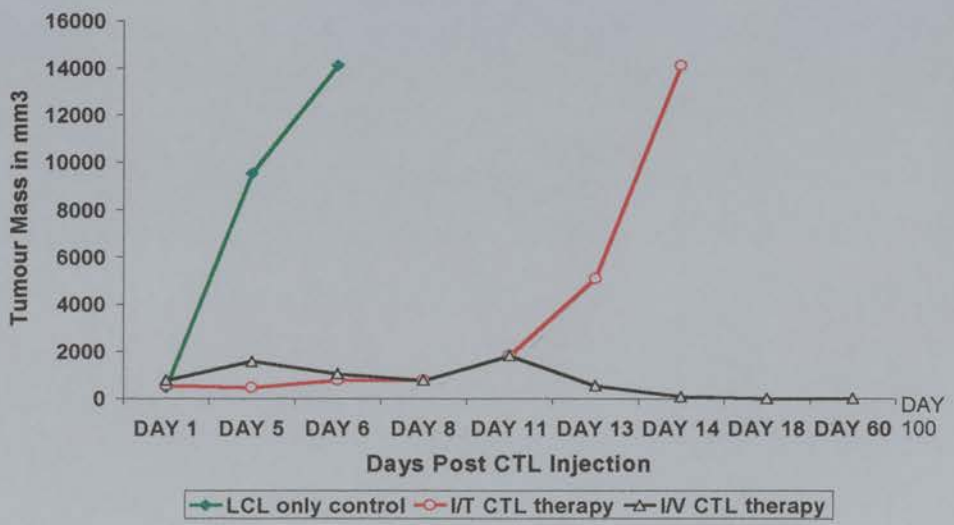


Figure 3.7

I/V and I/T Treatment of Subcutaneous LCL induced tumours in SCID mice with autologous polyclonal CTL from donor 4.

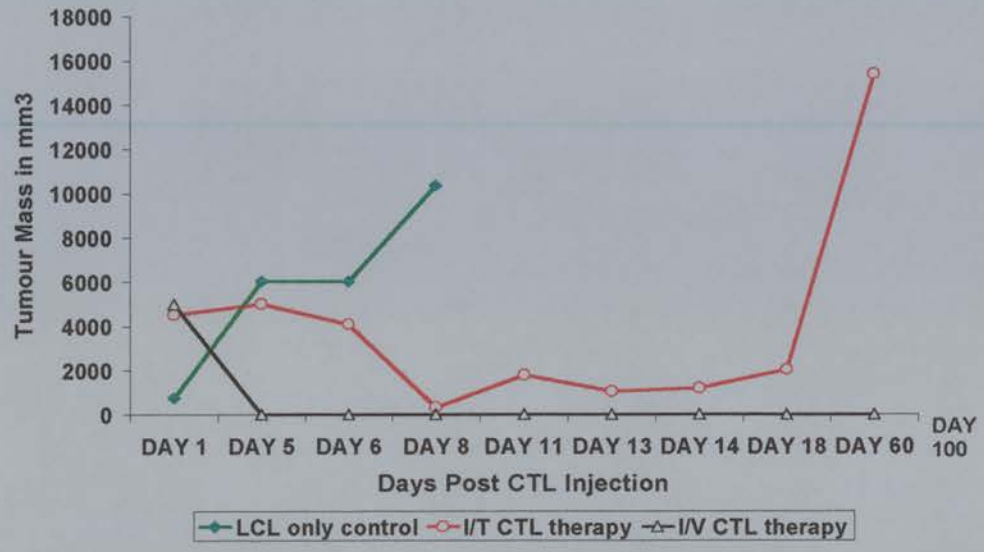


Figure 3.8

I/T & I/V Treatment of Subcutaneous LCL induced tumours in SCID mice with autologous polyclonal CTL in donor 5.

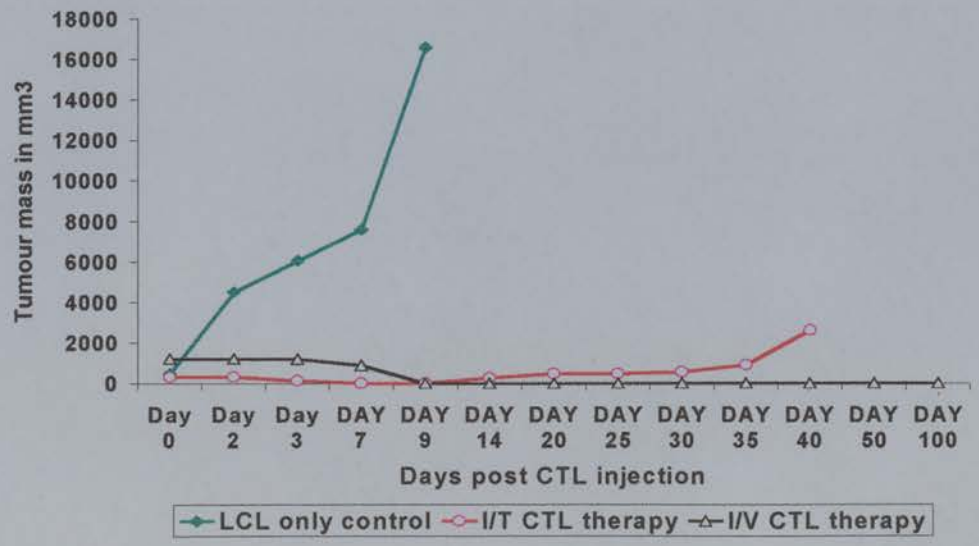
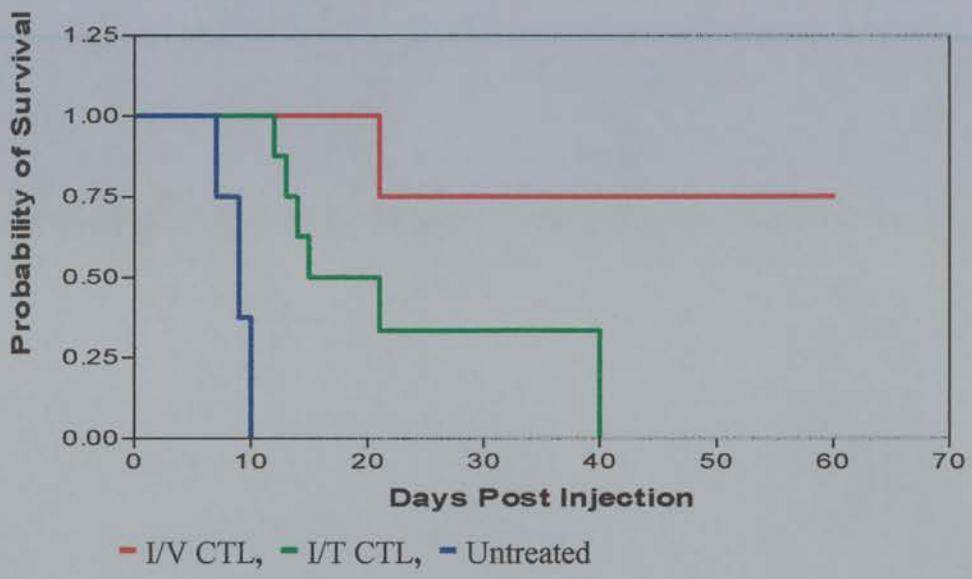


Figure 3.9

Comparison of survival curves for non-treated I/V treated and I/T treated animals

For each X value (time) the fraction still alive is shown. Survival fractions were calculated using the Kaplan-Meier method.



3.3.2 Therapy of established LCL induced Subcutaneous Tumours with Multiple Autologous CTL Infusions

Results of the previous experiment showed that reduction in tumour mass could be achieved with a single infusion of autologous CTLs and could cause total regression of gross tumour in some cases. Since I/V therapy appeared to be more efficient in causing tumour regression as compared to I/T CTL therapy, the next set of experiments was designed to test the efficacy of multiple (weekly) fixed dose autologous I/V CTL infusions for treatment of already established subcutaneous LCL tumours in the SCID model.

Single midline LCL tumours from donor 1, 2 and 5 were generated in three SCID mice each. In each group one mouse served as a non-treated LCL control and the other two were treated with weekly fixed dose (3×10^6 cells) I/V injections of autologous CTLs. Treatment was started after the appearance of gross tumours (Day 0) and I/V CTL injections were delivered weekly on day 1, 7 and 14. A reduction in the rate of increase of tumour mass was observed in all treated cases

as compared to the LCL only control groups. When animals from all three treated groups were considered collectively 67% of treated animals displayed a total disappearance of gross tumour between day 7 and 9. With CTL from donor 1 both treated animals displayed no change in size or rate of growth of tumour after the first injection for the first three days. SCID 1 showed a decrease in tumour mass on day 6 post-injection but the tumour did not respond to subsequent injections and the animal was sacrificed. SCID 2 did not show a change in tumour mass after the first CTL injection, however, complete regression of gross tumour was observed by day 14, one day after the delivery of third CTL infusion. The animal remained tumour free till end of experiment. Results are shown in figure 3.10.

With CTL from donor 2 decrease in the rate of tumour growth was observed in both treated animals after the first CTL infusion but the outcome was different in the two animals. In SCID 1 tumour mass stabilised after the first CTL infusion. Subsequent weekly CTL injections were delivered and the tumour mass remained stable until day 14 (third CTL infusion) but started to increase thereafter and animal was sacrificed on day 35. Tumour mass stabilized in SCID 2 after first CTL infusion and complete regression of tumour was observed by day 9. The animal remained tumour free till the end of the experiment. Results are shown in figure 3.11.

With CTL from donor 5 after the first CTL infusion both treated animals showed decrease in rate of increase in tumour mass as compared to the LCL only control. Complete reduction in gross tumour mass was seen in both animals by day 14, and they remained tumour free till the end of experiment. The results are shown in figure 3.12. Survival analysis using the Kaplan-Meier method showed that probability of survival remained between 0.75-1.0 for the treated group while it became 0.0 for the non-treated animals by day 15 after tumour appearance. Survival curve is shown in figure 3.13.

Figure 3.10

Response of LCL induced subcutaneous tumours in SCID mice to multiple fixed dose autologous CTL infusions from donor 1.

(Blue arrow indicates time of CTL injection)

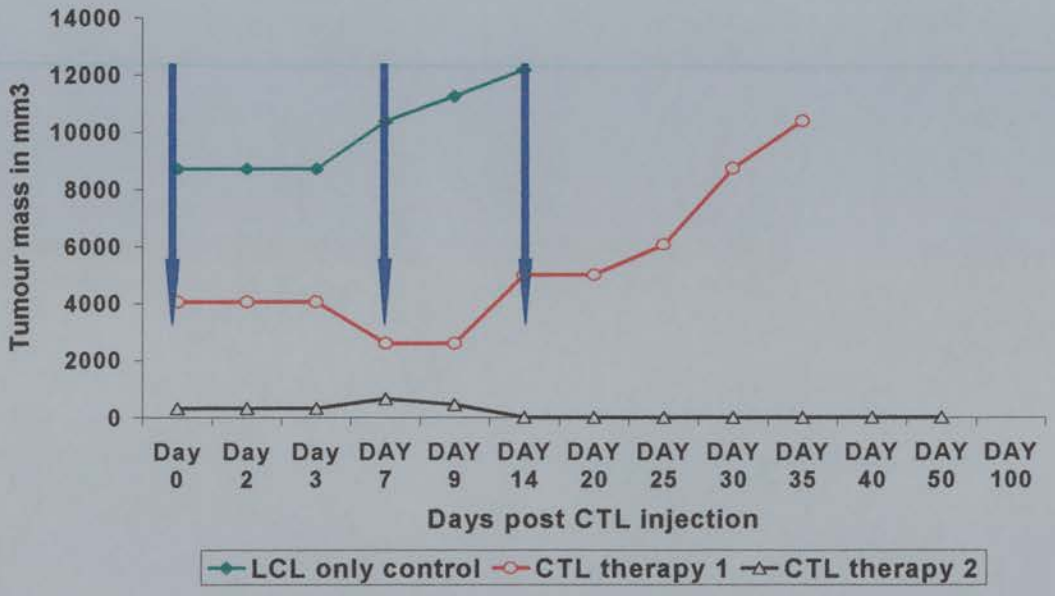


Figure 3.11

Response of LCL induced subcutaneous tumours in SCID mice to multiple fixed dose autologous CTL infusions in donor 2.

(Blue arrow indicates time of CTL injection)

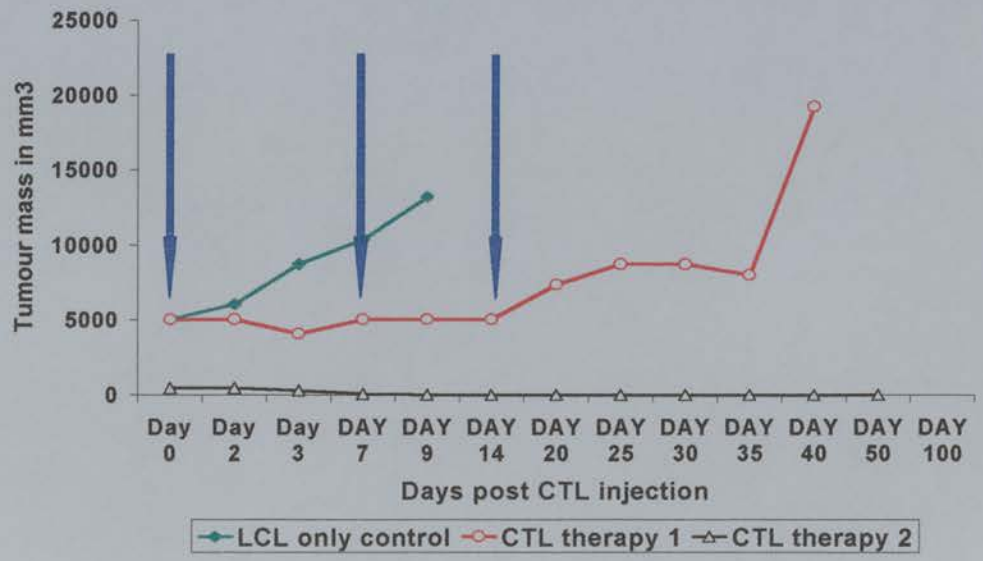


Figure 3.12

Response of LCL induced subcutaneous tumours in SCID mice to multiple fixed dose autologous CTL infusions in donor 5.

(Blue arrow indicates time of CTL injection)

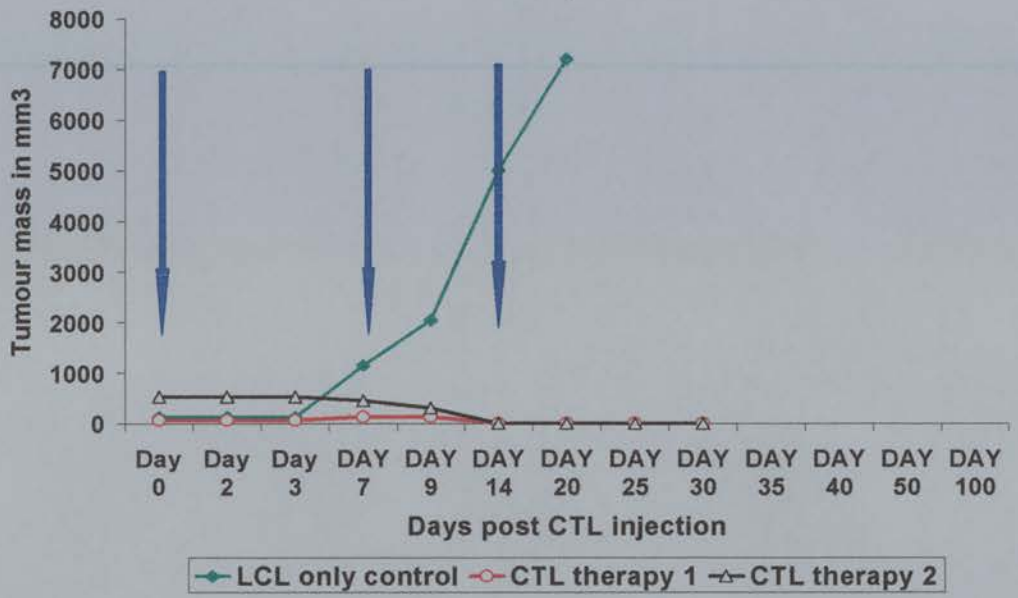
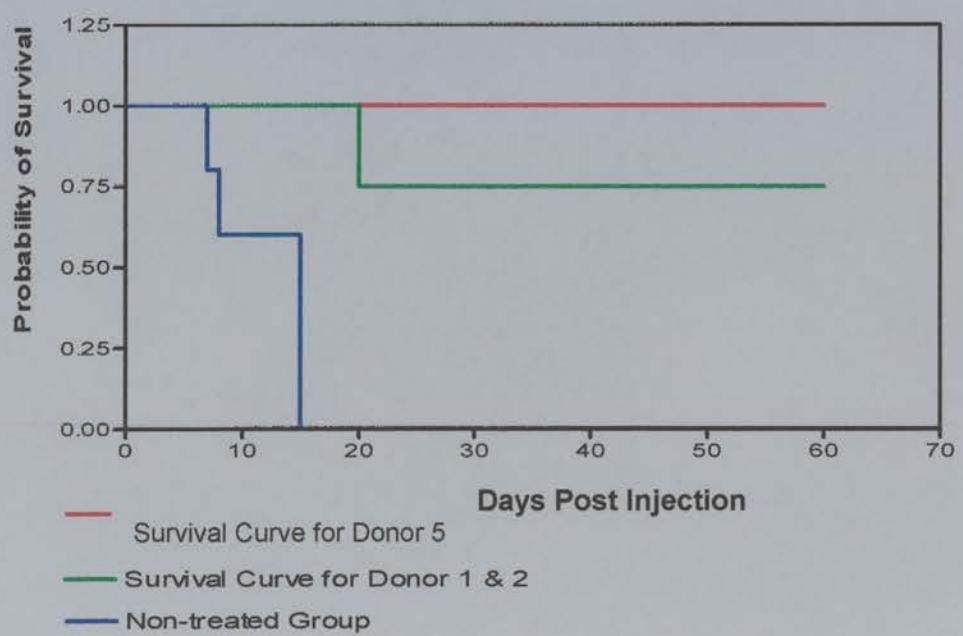


Figure 3.13

Survival analysis of treated and non-treated SCID groups after Multiple CTL Infusions

For each X value (time) the fraction still alive is shown. Survival fractions were calculated using the Kaplan-Meier method.



3.3.2.1 Conclusions

Results of these experiments showed that repeated fixed dose CTL injections were successful in causing regression of autologous LCL induced subcutaneous tumours in SCID mice. Collectively four out of six tumour bearing animals (67%) which were treated with autologous CTLs showed disappearance of gross tumour and were tumour free at the end of the experiment. Tumours that did not respond to CTL treatment were larger (tumour mass 4050 and 5000 mm^3) at the time of start of CTL therapy than those which responded (tumour mass 312 and 450mm^3).

4. RESULTS 2

4.1 Standardization of Non-isotopic In Situ Hybridization Technique for Cytokine mRNA Detection in EBV Related Diseases

The aim of this study was to investigate the cytokine profile of EBV positive cells in normal lymphoid tissue and EBV related malignancies by non-isotopic in situ hybridization and to identify the cell type(s) responsible for cytokine production. The cytokines probed for included Interleukin (IL)-2, 4, 6, 10 and interferon gamma (INFγ). Cytokine mRNA was detected using commercially available 3' and 5' Digoxigenin (DIG) labeled oligonucleotide probe cocktails. β-actin was included as a positive control for mRNA detection and PHA stimulated human PBMC were used as positive controls for cytokine mRNA detection. Negative controls included no probe sections and RNase treated sections and specificity of the technique was tested by inclusion of mouse tissue. The technique was used to probe paraffin embedded tissue from human PBMC-induced I/P tumours in SCID mice, EBV related malignancies: PTLD, NPC and HD and a tonsil from an I/M patient .

The NISH protocol used in this study was a modification of a previous protocol used to detect IFNγ mRNA transcripts in formalin fixed wax embedded allergen stimulated skin biopsies (Howie et al, 1996). This protocol was modified and re-standardized for the current study. The modified technique (protocol 3, table 2.5) was first applied to formalin fixed paraffin embedded allergen-stimulated skin sections to detect INFγ mRNA expression. The sections stained positive for INFγ mRNA transcripts and results are shown in figure 4.1 which shows both a positive and a no-probe section.

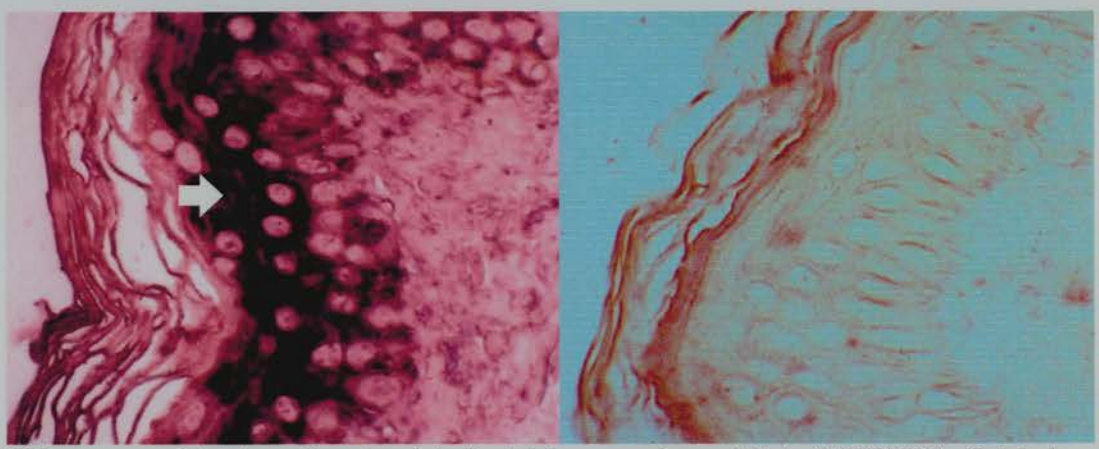
With protocol 3 PHA-stimulated blasts consistently gave a positive signal for all cytokines tested and were used as positive controls for cytokine mRNA detection in the study. Results are shown in figure 4.2 a and b. Negative controls (no probe and RNase treated sections) are shown in figure 4.2a. RNase treated sections received one type of probe per NISH run (IL-2). For specificty control paraffin embedded SCID mouse liver and spleen tissue was included with each NISH run.

Inclusion of mouse tissue also checked the cross reactivity of cytokine probes with mouse cytokines (figure 4.2a).

Figure 4.1

In situ detection of INFγ mRNA in allergen stimulated skin tissue by NISH technique

a, Positive INFγ Signal **b, No Probe Negative Control**

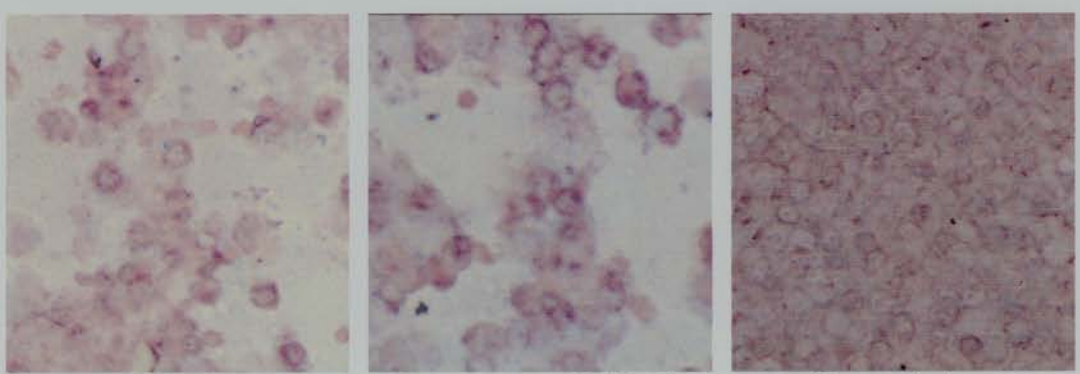


White arrow points at positive signal, indicated by a purple precipitate (BCIP/NBT). Original Original magnification x400

Figure 4.2

Detection of Cytokine mRNA in formalin-fixed human PHA-stimulated blast cells.

4.2a Negative controls

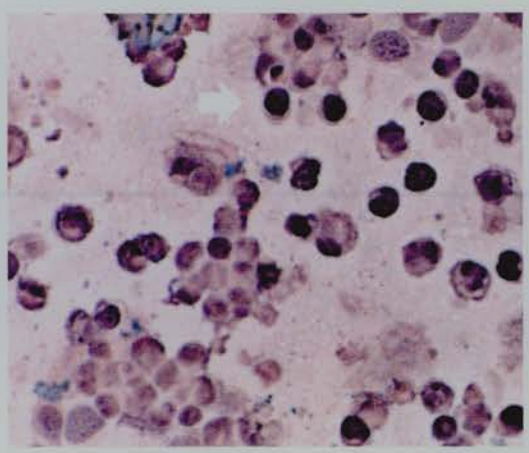


No Probe **Rnase treated (IL-2)** **Mouse Spleen**

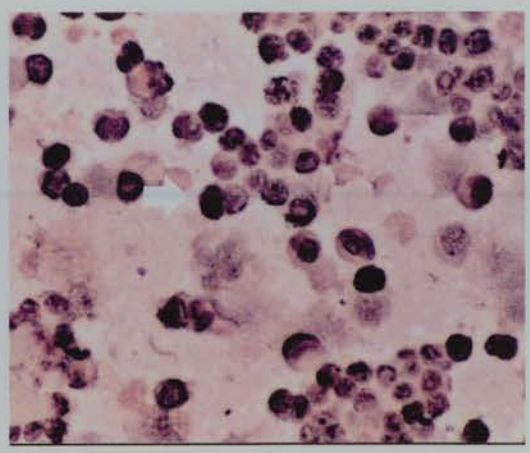
Original magnification x200

4.2b Cytokine mRNA Expression in PHA stimulated PBMC

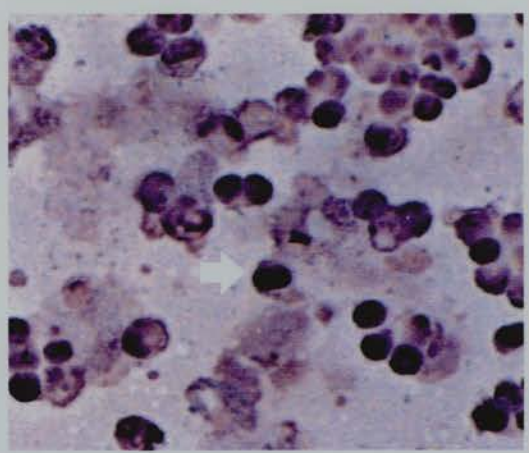
Original magnification of all sections x200. Arrow indicates some of the positive cells



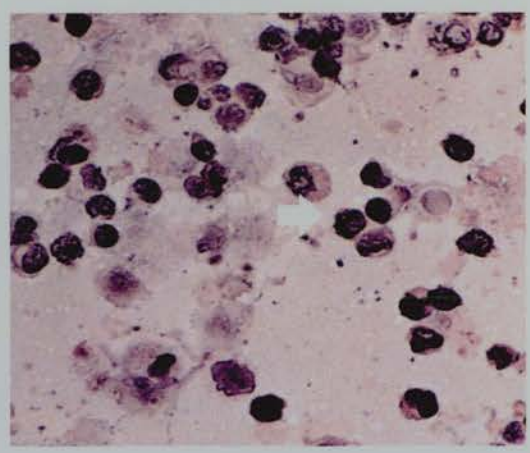
β-actin



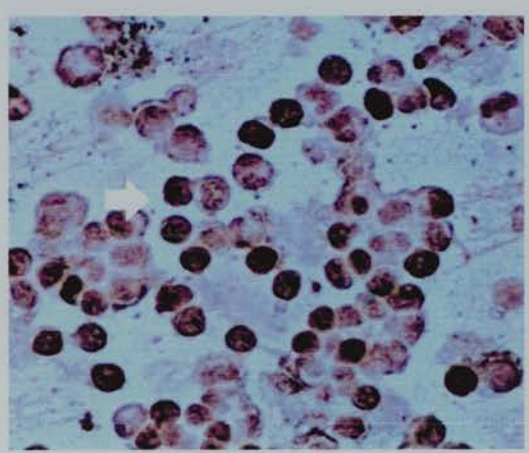
IL-2



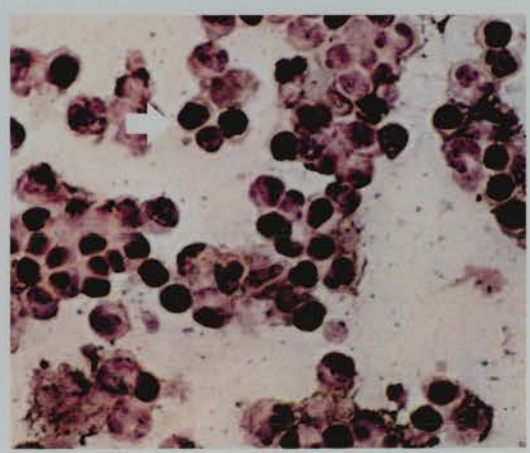
IL-4



IL-6



IL-10



IFNγ

Paraffin embedded sections were dewaxed and rehydrated as described in the materials & methods (section 2.9.2). Signal to noise ratio (specific to non-specific staining) was enhanced by incubating slides in 2 x SSC at 60^0C for ten minutes before hybridisation. Omitting this step or a change in temperature or time resulted in increased non-specific staining.

For unmasking the mRNA signal, sections were treated with an optimum concentration of PK (10ug/ml for 10 minutes at 37^0C). Overdigestion of sections was visible as tissue detachment from slides, vacuolated areas within sections and intensely staining nucleoli, which are resistant to PK digestions.

Cytospin preparations and wax embedded sections were incubated with 20ng of each probe for 18-24 hours at 37^0C in a humid chamber. Stringency washes were carried out as described in the materials and methods section. Sections were also incubated in 2-20% sheep, rabbit and donkey serum for ten minutes to block non-specific binding of primary, secondary and tertiary antibodies.

Signal detection and amplification

Three protocols were followed to detect and enhance the detection of cytokine mRNA signal (Table 2.5, section 2). Protocol 1, in which an anti-digoxigenin antibody coupled with alkaline phosphatase (AP) was used for probe detection did not give any signal in all cases. With protocol 2 mRNA signal was detected in some sections but was not strong enough for adequate interpretation. To enhance the signal, in protocol 3 another amplification step was introduced by including a FITC labelled and an anti-FITC antibody. Addition of this step enhanced cytokine mRNA signal without resulting in non-specific staining. BCIP/NBT was the AP substrate of choice for in situ hybridization because it gave a good localization of signal and had a high sensitivity.

Sections were observed under a light microscope to examine the intensity and distribution of mRNA signal. The signal was reported by counting the number of positive cells per field and 15-20 fields per section were counted. Results were reported in a semiquantitative way as described in table 4.1

Table 4.1

Semiquantitative Measurement of Cell Numbers Positive for Cytokine mRNA Signal

-	-	negative,
-	+/-	< 1% positive cells
-	+	1-5% positive cells
-	++	5-20% positive cells
-	+++	20-50% positive cells
-	++++	>50% positive cells

15-20 fields were counted per section under x 200 magnification.

4.1.1 Detection Of Cytokine mRNA in EBV Positive Tumours from SCID Mice

Human PBMC from EBV positive donors result in human B cell tumours when injected I/P into SCID mice. These tumours mimic EBV positive PTLD in humans and serve as a pre-clinical murine model for PTLD. Cytokine mRNA expression of ten such tumours was investigated using NISH. The cell type responsible for cytokine production and the relationship to EBV status was tested by staining serial sections for CD3 (T cell marker), CD20 (B cell marker) and EBER expression. The number of cytokine positive cells was reported as percentage of β-actin positive cells expressing cytokine mRNA.

Although cytokine mRNA was detected in most cases, the number of cells positive for different cytokines varied between different tumours. Notably tumours which gave a poor signal for β-actin mRNA (tumours No. 6, 9 and 10) failed to show appreciable signal for cytokine mRNA. Cytokine positive areas predominantly equated with those of CD20 and EBER positive cells. This was suggestive of predominant autocrine cytokine production by tumour cells themselves. All negative controls remained negative for mRNA signal. Results are presented in table 4.2. Figure 4.3 shows SCID tumour no 1 which stained positive for all the cytokines in the panel and serial sections stained for CD20 and EBERs.

Table 4.2

Cytokine mRNA Production by EBV Positive Human PBMC Induced Tumours in SCID Mice

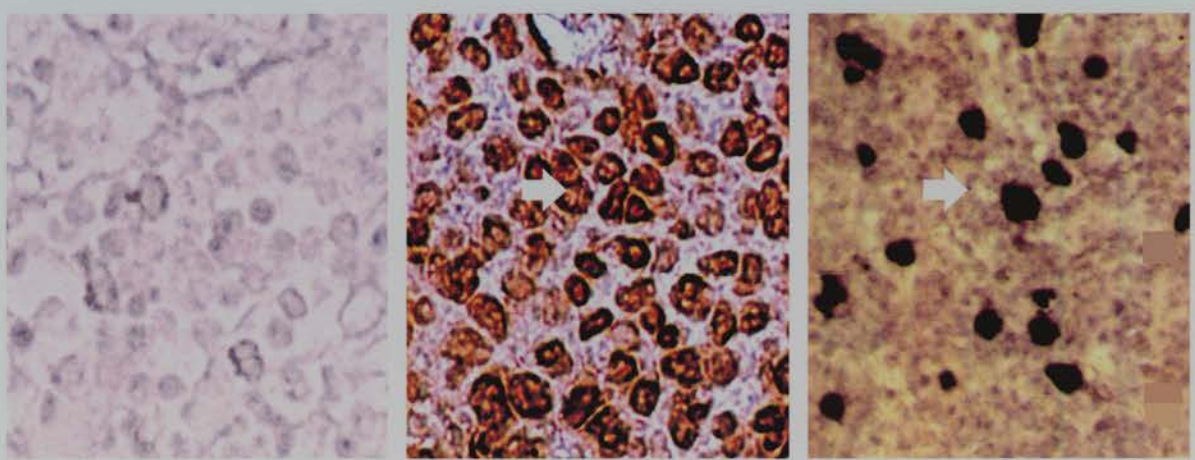
Tumour	Cytokines					
	β-actin	IL-2	IL-4	IL-6	IL-10	INFγ
1	++++	++++	+++	+++	+	++++
2	++++	++++	-	++++	++++	++++
3	+	++	+	-	+/-	+/-
4	+++	NT	++	+++	+++	+++
5	++	+++	++	-	+	+
6	+	-	-	-	+	-
7	++++	+++	-	+++	++++	+++
8	+++	+	+	+++	-	-
9	+	-	NT	-	-	-
10	+	-	-	-	-	-

Number of cytokine positive cells is the percentage of β-actin positive cells expressing cytokine mRNA. Positive control (PHA Blasts) were positive for mRNA of all cytokines tested. No probe, RNase digested human tissue and mouse tissue were negative

Figure 4.3

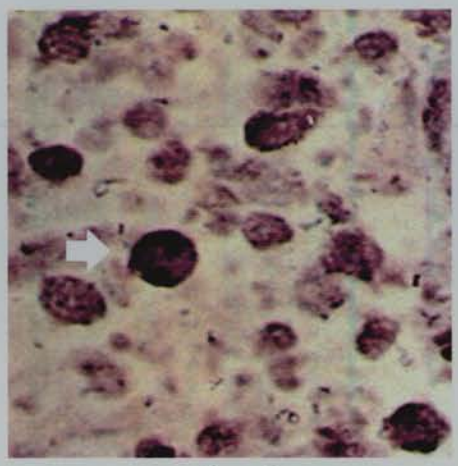
CD20 Staining, EBER and Cytokine mRNA Detection in a Human PBMC-Induced EBV-Positive Tumour in SCID Mouse (Tumour No. 1)

4.3a No probe control, CD20 staining and EBERS signal in SCID tumour 1 (Original Magnification x200)

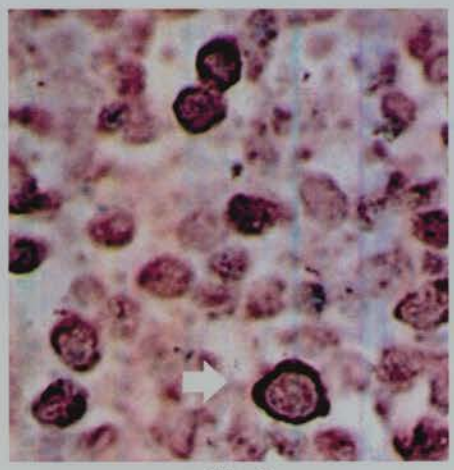


No Probe Section **CD20 Staining** **EBERS**

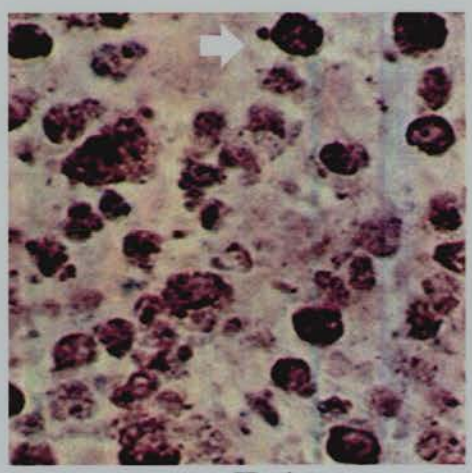
4.3b Results of NISH for EBERS and Cytokine gene transcripts on SCID tumour 1 (Original Magnification x 400. Arrows point at some of the positive cells)



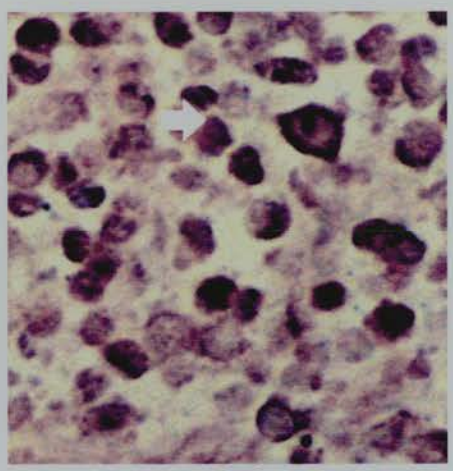
β-actin



IL-2

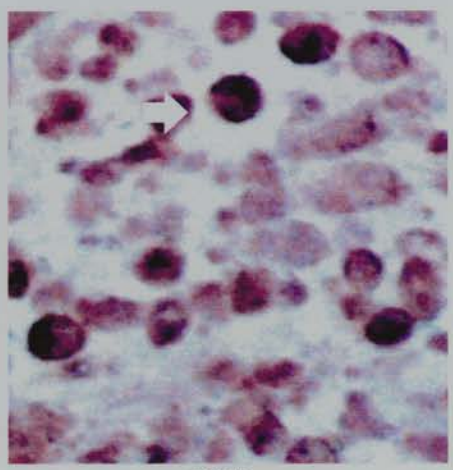


IL-4



IL-6

IL-10



IFNγ

4.1.2 Detection Of Cytokine mRNA In EBV Positive PTLD Tumours

The cytokine mRNA profile of 13 PTLD tumours was studied using the NISH technique. Paraffin embedded blocks were kindly supplied by Dr. J A Thomas, Department of Infection and Immunity, London School of Hygiene & Tropical Medicine, London and Dr P Amlot, Department of Immunology, Royal Free Hospital School of Medicine, London. Blocks were cut into 6um sections, mounted on silane coated slides and probed for the IL-2, 4, 6, 10 and INFγ mRNA. Serial sections from all tumours were probed for EBER RNA and stained for CD3 and CD20. In situ detection of β-actin mRNA served as a positive control for mRNA detection and tumours with lower number of β-actin positive cells (tumour 10) showed lower number of cytokine positive cells than tumours which showed higher number of cells positive for β-actin mRNA. Apart from tumour number 10, all tumours showed cells positive for transcripts for all cytokines in the panel. Negative controls including RNase treated sections, no-probe sections and mouse tissue did not stain for cytokine mRNA. > 80% cells in all tumours were positive for EBER mRNA and CD20. Less than 1% cells stained for CD3. Positive areas for cytokine transcripts equated with those of CD20 and EBER positive cells, indicative of autocrine cytokine production by tumour cells. Results are shown in table 4.3 and figure 4.4.

Table 4.3 In situ Cytokine mRNA Detection in PTLD Tumours

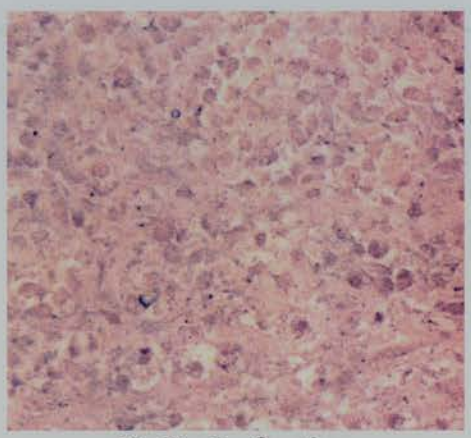
PTLD	CYTOKINES						
Pt. No.	β-Actin	IL-2	IL-4	IL-6	IL-10	INFγ	Neg.
1	+++	+	++	+++	+++	++	-
2	++	+	+	++	+	++	-
3	++	++	++	++	++	++	-
4	++	++	+	++	+	++	-
5	++	+	++	+	++	++	-
6	++	+	++	+++	+++	+	-
7	++	NT	+	++	++	+	-
8	++	+	++	+/-	+	+	-
9	++	+	+/-	++	+	++	-
10	+/-	-	-	-	+	+/-	-
11	++	+	++	++	++	++	-
12	++	++	++	++	++	+	-
13	++	++	++	++	++	NT	-

NT: Not Tested

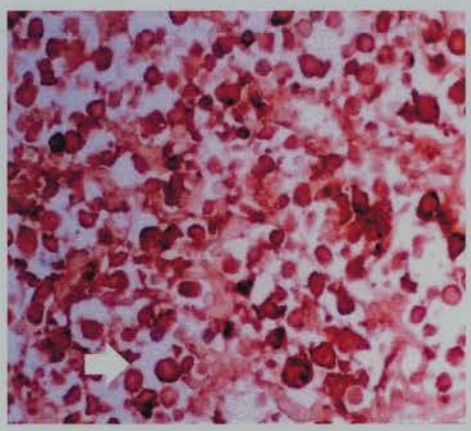
Figure 4.4

Cytokine and EBER mRNA Detection and CD20 Staining of a PTLD Tumour

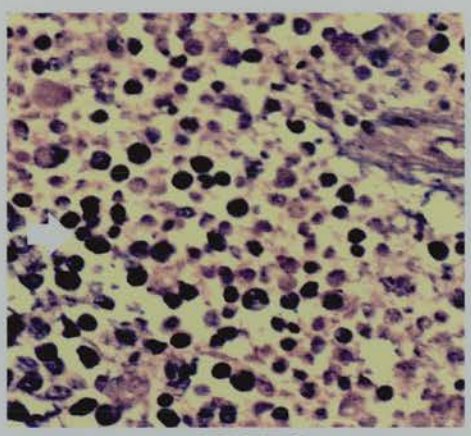
Original magnification x200. Arrows indicate positive cells



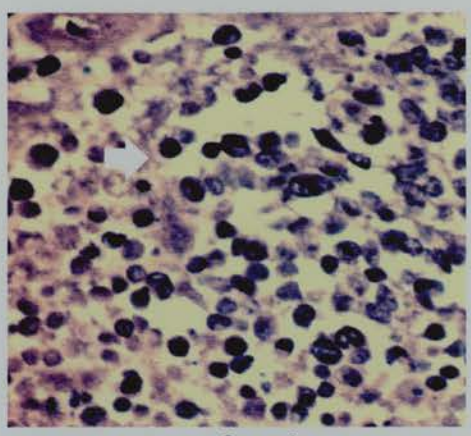
No Probe Section



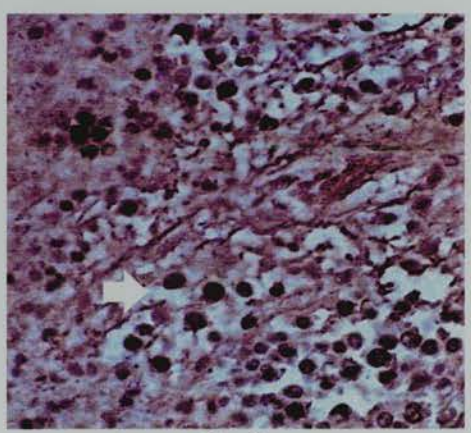
CD20



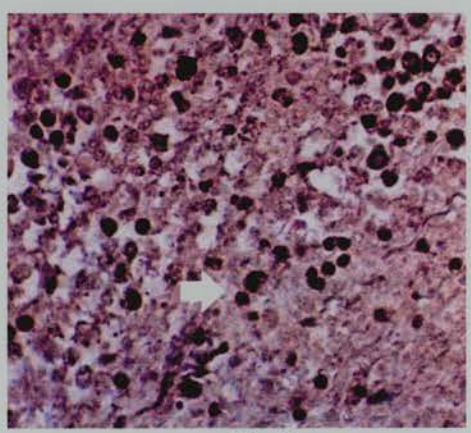
EBERS



β-actin

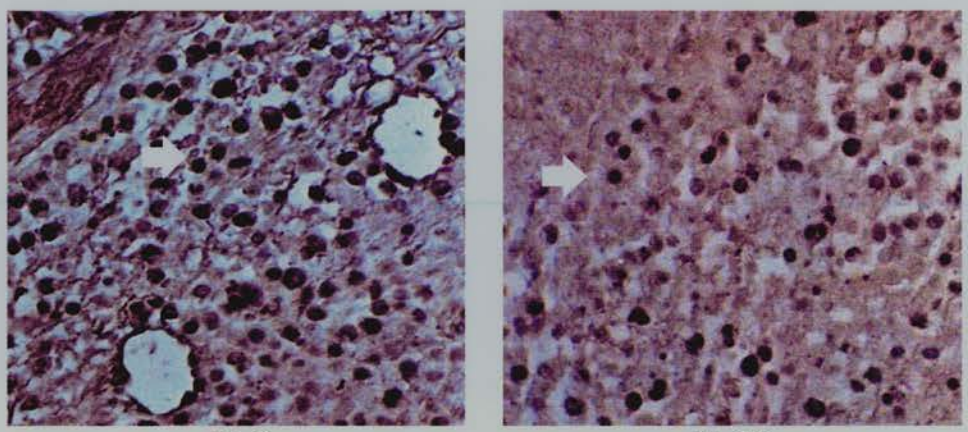


IL-2

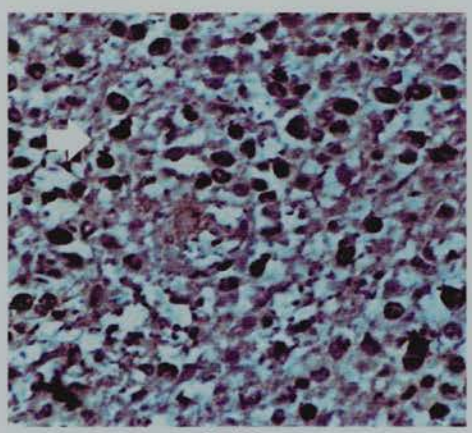


IL-4

Figure 4.4



IL-6 IL-10



IFNγ

4.1.3 Detection Of Cytokine mRNA In EBV Positive NPC Tumours

Six paraffin embedded NPC tumour biopsies were kindly provided by Dr J A Thomas, London School of Hygiene & Tropical Medicine. Tumour sections were probed for IL-2 4, 6, 10 and INFγ mRNA. Tumours were positive for transcripts of all the cytokines on the panel, with a higher number of cells positive for IL-6 and IL-10 (5-20% cells). Negative controls which included no probe sections, RNase treated sections and mouse tissue remained negative for cytokine mRNA. Serial sections from each tumour were stained for EBERs, cytokeratin (epithelial tumour cell marker) and CD3 (infiltrating T cell marker) expression. Cytokine signal was observed both in tumour and infiltrating CD3 positive cells with a higher number of infiltrating T cells positive for cytokine transcripts than epithelial tumour cells. The results of cytokine mRNA detection in tumour cells

and CD3 cells are separately detailed in table 4.4 a and b. EBER signal was only observed in the malignant epithelial cells. Figure 4.5 shows one NPC tumour (No. 5) stained for cytokine and EBER mRNA, and cytokeratin and CD3 cell surface markers.

Table 4.4

a, Cytokine mRNA Detection in Infiltrating CD3 Positive Cells in EBV positive NPC tumours

NPC	CYTOKINES						
Pt. No.	β-Actin	IL-2	IL-4	IL-6	IL-10	INFγ	Neg.
1	++	+	+	++	++	-	-
2	+	+/-	-	-	+/-	-	-
3	++	+	-	++	++	-	-
4	++	+	+	++	++	+	-
5	++	+	++	++	++	+	-
6	+	-	+/-	+	+	+	-

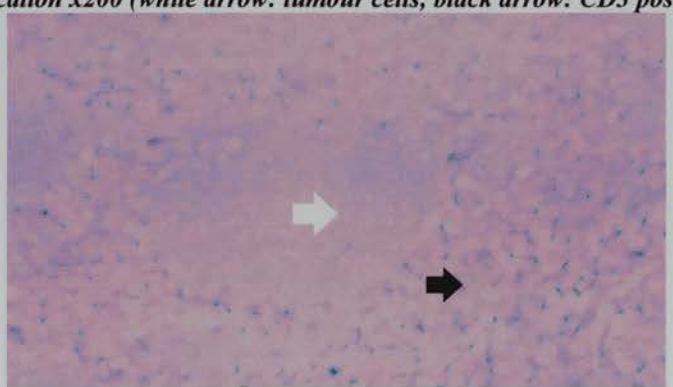
b, Cytokine mRNA Detection in EBER Positive Malignant Cells in EBV positive NPC tumours

NPC	CYTOKINES						
Pt. No.	β-Actin	IL-2	IL-4	IL-6	IL-10	INFγ	Neg.
1	++	+	-	++	++	-	-
2	+	+/-	-	-	+/-	-	-
3	++	+/-	-	+	+	-	-
4	++	+	-	+	+	-	-
5	++	-	+	++	++	-	-
6	+	-	-	+	+	-	-

Figure 4.5

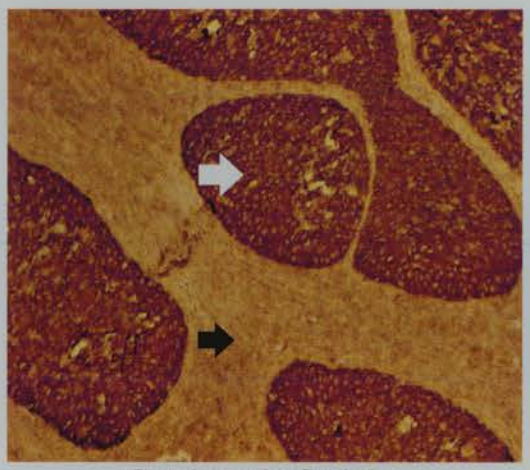
Cytokine and EBER mRNA Detection and Cytokeratin and CD3 Staining of EBV Positive NPC Tumours

Origianl magnification x200 (white arrow: tumour cells, black arrow: CD3 positive cells)

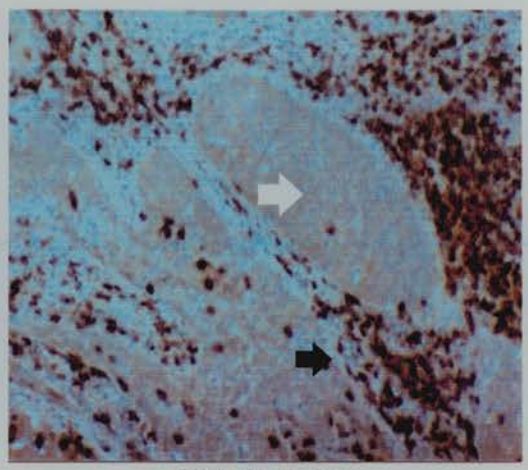


No Probe Section

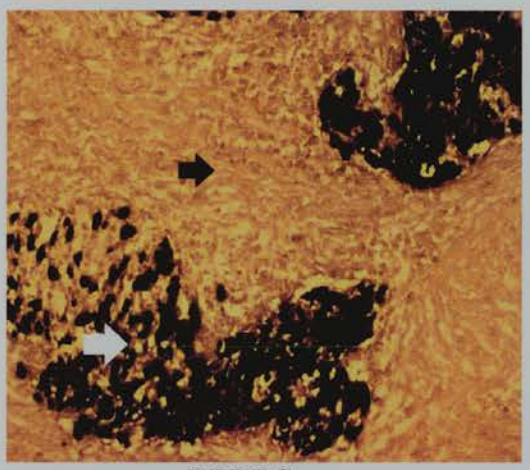
Figure 4.5



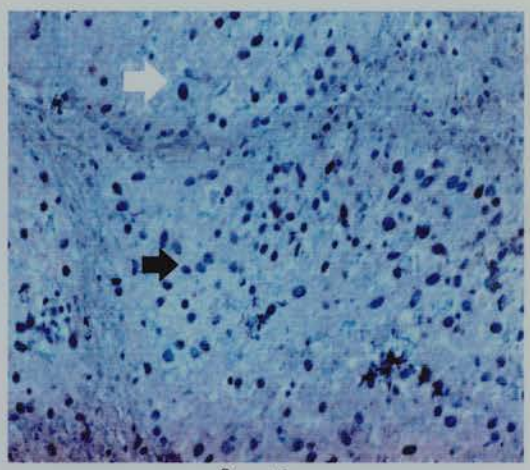
Cytokeratin Staining



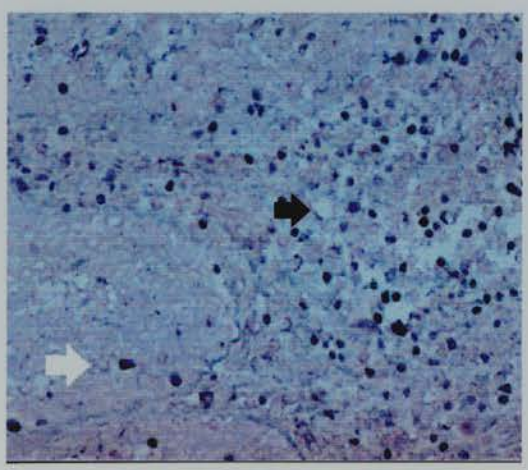
CD3 Staining



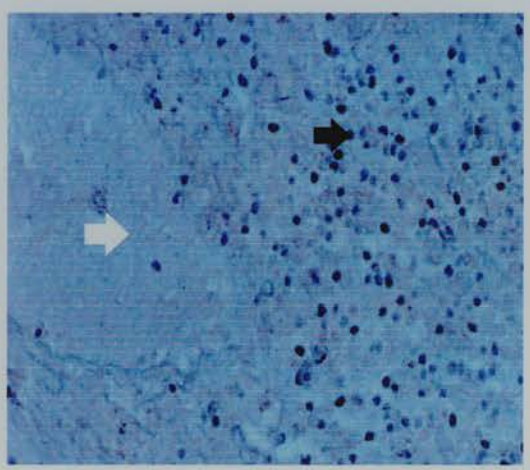
EBERS



β-actin

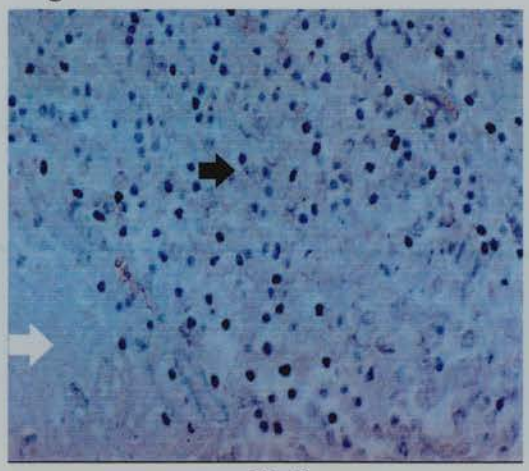


IL-2

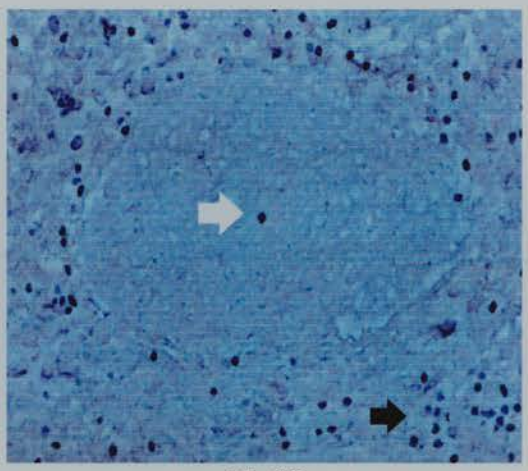


IL-4

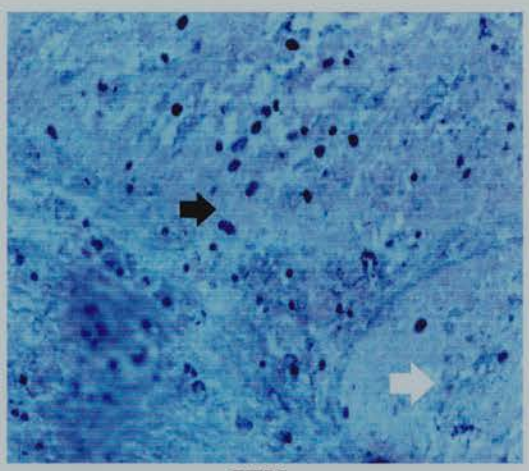
Figure 4.5



IL-6



IL-10



IFNγ

4.1.4 Detection Of Cytokine mRNA In EBV Positive HD

Five EBV positive HD tumours, kindly provided Dr A Krajewski from Department of Pathology, University of Edinburgh, were stained for expression of IL-2, 4, 6, 10, INFγ, EBERs and R/S cell specific markers: CD15 and 30. Detection of β-actin transcripts served as a positive control for mRNA detection. Positive cytokine controls included PHA stimulated blasts. Negative controls included no probe tumour sections, RNase-treated sections and mouse tissue.

Sections were necrotic and only 1%-20% cells stained positive for β-actin transcript and cytokine signal was only observed in cells within the β-actin positive areas. Cytokine transcripts were detected in 1-5% cells in all tumours and

cytokine and EBERs signal was mainly limited to CD30 and CD15 positive cells, of which over 50% were positive. Only tumour number 3 tested positive for all cytokines. Due to poor quality of sections and low β-actin signal the results were not interpretable. Results are shown in table 4.5 and figure 4.6.

Table 4.5

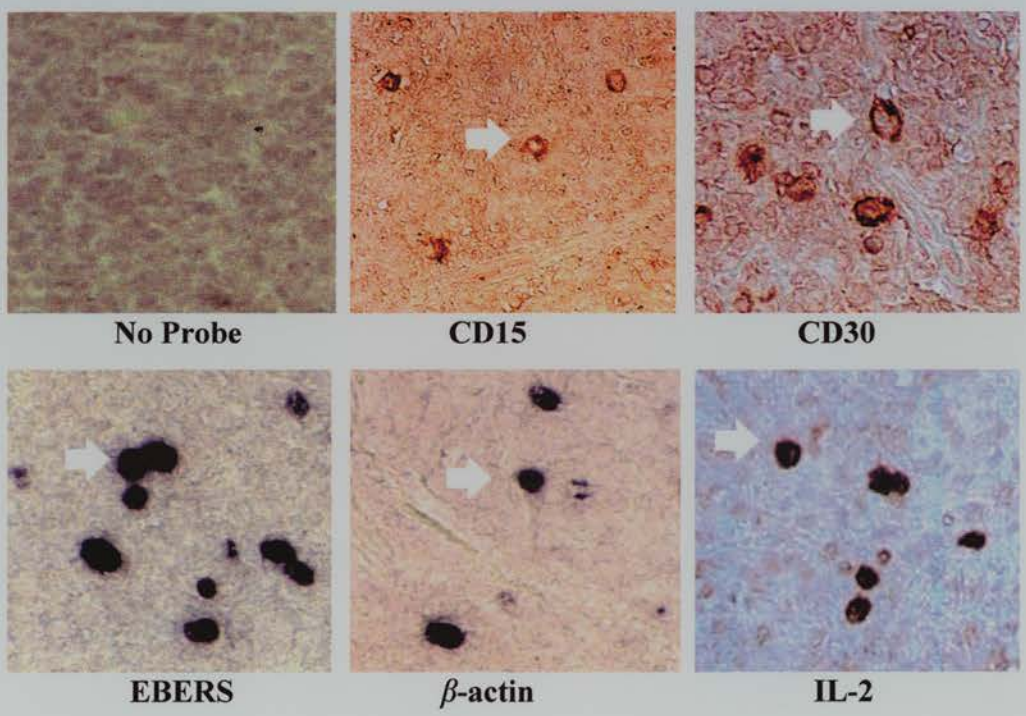
Cytokine mRNA Expression in EBV Positive HD

HD	CYTOKINES						
Pt. No.	β-actin	IL-2	IL-4	IL-6	IL-10	1NFγ	Neg
1	+/-	-	-	-	-	-	-
2	++	+	+	-	+	+	-
3	++	+	+	+	+	+	-
4	+	+	-	+	+	-	-
5	+	-	+	+	-	+	-

Figure 4.6

Cytokine, EBER, CD15 and CD30 Staining of EBV Positive HD (tumour no. 3)

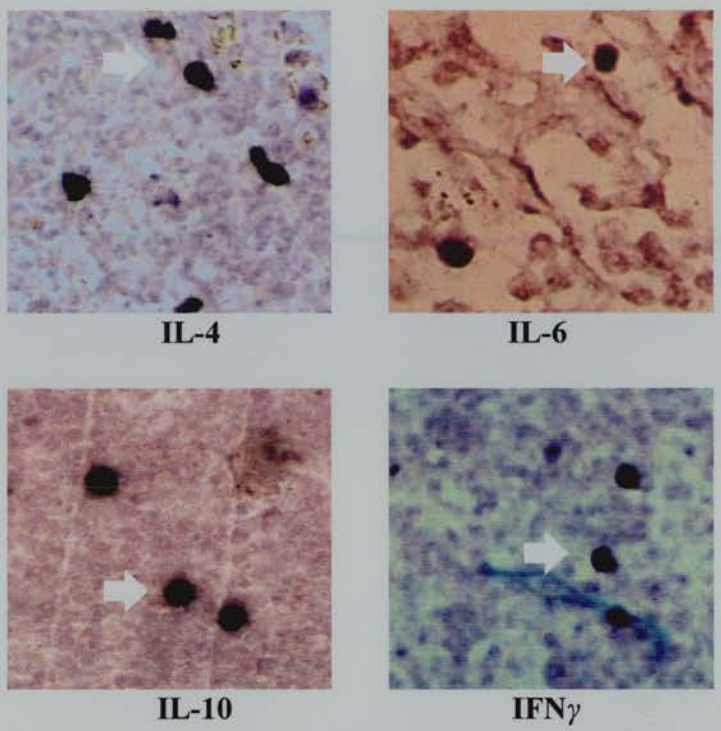
Original magnification x400. Arrows indicate positive cells



No Probe **CD15** **CD30**

EBERS **β-actin** **IL-2**

Figure 4.6



4.1.5 Detection Of Cytokine mRNA in Tonsil From an Acute I/M Patient

Paraffin embedded tonsil tissue from an acute IM case was kindly provided by Dr A. Krajewski, Department of Pathology. Sections were probed for IL-2, 4, 6, 10 , INFγ and EBER transcripts. Positive controls included β-actin and PHA stimulated blasts. Negative controls included no probe and RNase treated sections. Tissue tested positive for all cytokines. 20% cells stained positive for IL-6 and 10. 1-5% cells gave a positive signal for IL-2 and less than 1% cells stained positive for INF-γ and IL-4. Serial sections were stained positive for EBERs and the cells positive for EBERs signal equated with those of cytokine production. Results are shown in Table 4.6 and figure 4.7.

Table 4.6

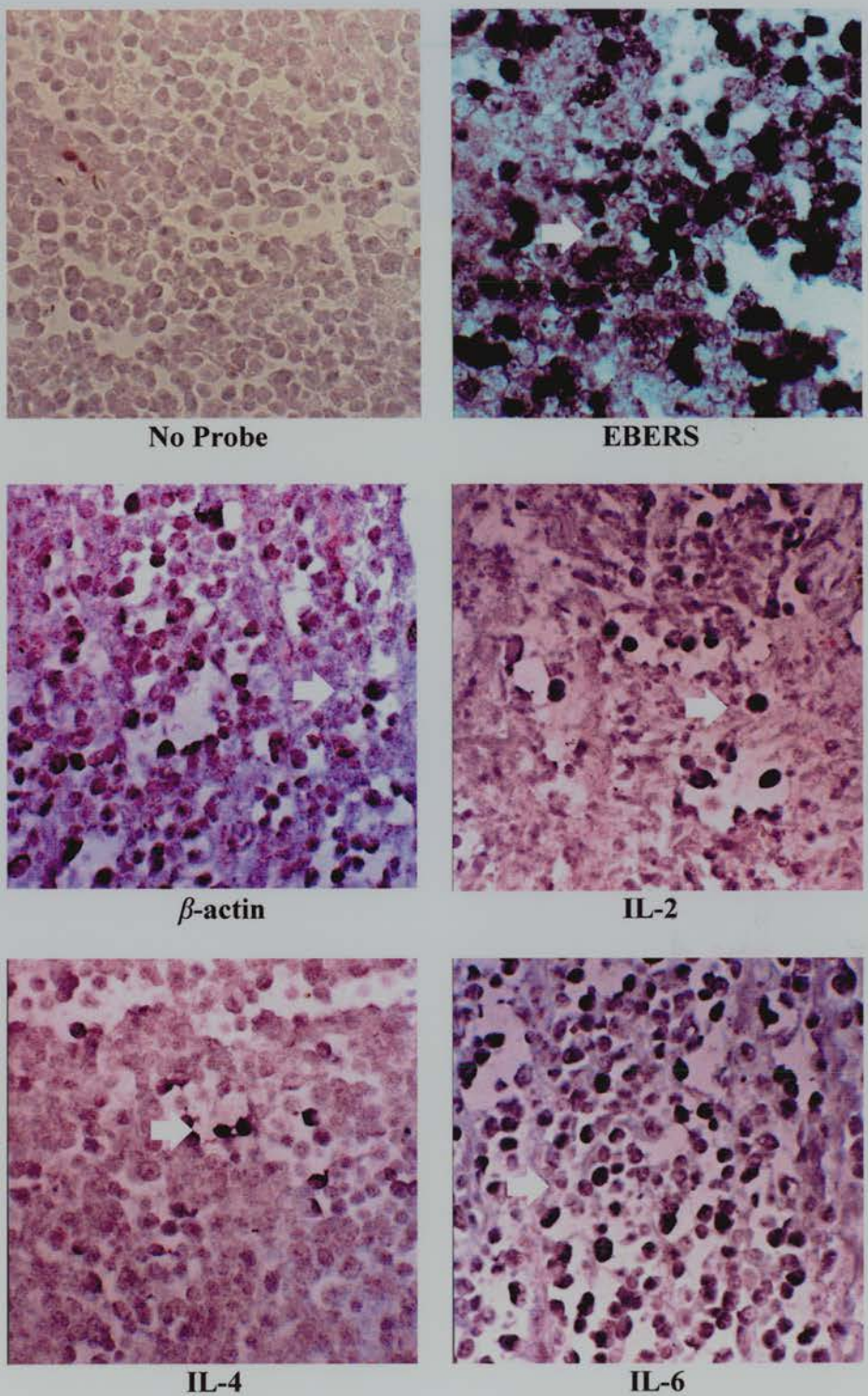
Cytokine mRNA detection in tonsil sections from an acute case of Infectious Mononucleosis

	EBERs	β-Actin	IL-2	IL-4	IL-6	IL-10	INFγ	Neg.
Pt	+	++	+	+/-	++	++	+/-	-

Figure 4.7

Cytokine and EBER mRNA Detection in Tonsillar Tissue from an I/M Patient

Original magnification x200. Arrows indicate positive cells.

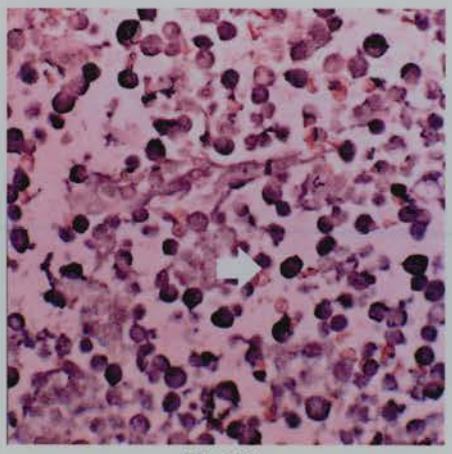


No Probe **EBERS**

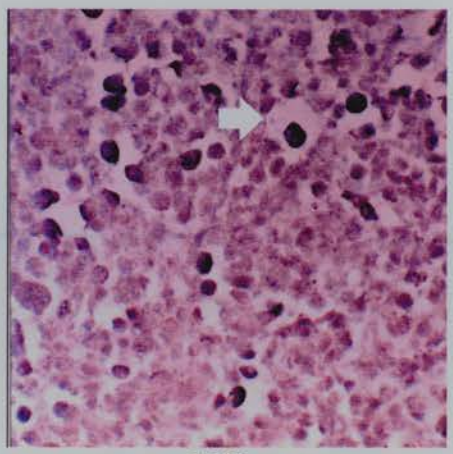
β-actin **IL-2**

IL-4 **IL-6**

Figure 4.7



IL-10



IFNγ

5. Discussion

5.1 Adoptive Cellular Immunotherapy of EBV Related Lymphoproliferative Disease in the SCID Mouse Model

EBV associated PTLD is an important clinical problem in transplant recipients receiving immunosuppressive therapy for prevention of allograft rejection. In individuals with normal immune responses, EBV infected B cells are kept in check by EBV-specific CTLs (Rickinson & Moss, 1997). However, immunosuppression in transplant recipients reduces the EBV-specific CTL activity and can result in uncontrolled expansion of EBV infected B cells leading to PTLD (Penn, 1987; Sokal et al, 1997). PTLD is conventionally treated with reduction or withdrawal of immunosuppressive drugs and/or delivery of cytotoxic drugs such as vincristine and corticosteroids (reviewed in Kawa, 2000). These treatment modalities can cause tumour reduction in some PTLD cases but can result in graft rejection and/or toxic side effects.

Cell mediated immunity plays a critical part in controlling EBV infection in the normal host and in recent years the potential use of EBV-specific CTLs in treatment of EBV related PTLD has been investigated by several research groups. Previously adoptive transfer of PBMC and *in vitro* expanded EBV-specific CTLs from EBV seropositive bone marrow transplant donors have been successfully used to control EBV associated PTLD tumours in transplant recipients (Papadopoulos et al, 1994; Heslop et al, 1994; Rooney et al, 1995; Heslop et al, 1996). Heslop et al have also shown that EBV-specific CTLs not only restore the cellular immune response to EBV in the short term but also induce CTL precursors which respond to *in vivo* and *in vitro* challenge with virus for up to 18 months post infusion (Heslop et al, 1996). The results of these previous studies suggest a definite role of cellular immunotherapy in controlling PTLD and support wider use of EBV-specific CTLs in both BM and solid organ transplant recipients.

Until recently, immunotherapy of PTLD was restricted to BMT recipients. This was because PTLD in BMT patients is of donor cell origin and since the donors are available and not immunosupressed, EBV-specific CTLs can be successfully expanded *in vitro* from donor PBMC for treatment of PTLD in the recipient. In solid organ transplant recipients the tumours are mostly of recipient B cell origin (Thomas et al, 1991) and *in vitro* activation of CTLs from these patients is difficult because of the effects of immunosuppression on the cellular immune response. However, recently Khanna et al, have reported *in vitro* activation of autologous CTLs from solid organ transplant patients undergoing primary EBV infection and have successfully used these cells to treat PTLD in one case (Khanna et al, 1999). This data is encouraging because it suggests that EBV-specific CTLs can be generated from recipient CTLs and thereby increases the scope of adoptive immunotherapy to PTLD in solid organ transplantation.

To further the knowledge on pathogenesis of PTLD, and to test new treatments, the pre-clinical SCID mouse model has been used regularly in recent years (Johannessen & Crawford, 1999; Boyle et al, 1993; Rencher et al, 1994). I/P injection of LCLs or PBMC from some EBV seropositive donors into SCID mice results in immunoblastic B cell lymphomas within a period of few weeks (Mosier et al, 1988, 1990, 1991; Ramqvist et al, 1991). The tumours are EBV positive, have a type III EBV gene expression pattern (Rowe et al, 1990) and have similar characteristics to PTLD in transplant recipients (Rowe et al, 1991, Gratama et al, 1991). These properties make the SCID mouse an effective model to investigate the efficacy of various treatment modalities and tumour vaccines before using them in humans. In our study we used the SCID model to investigate the effect of different autologous immune effector cells (unmanipulated PBMC, *in vitro* expanded EBV-specific polyclonal and epitope-specific CTLs) on outgrowth and treatment of LCL induced S/C tumours in the SCID model.

Although in humans Papadopoulos et al have reported successful control of PTLD by adoptive transfer of donor leukocytes to graft recipients (Papadopoulos et al, 1994), Lacerda et al reported no significant *in vivo* anti-tumour activity of unfractionated autologous PBMC in the SCID model (Lacerda et al, 1996). To

investigate if autologous unmanipulated PBMC would affect the outgrowth of EBV positive tumours, we monitored the effect of varying doses of autologous unfractionated PBMC on outgrowth of S/C LCL induced tumours in SCID mice. Our hypothesis was that PBMC from EBV seropositive donors contain EBV-specific CTLs which could potentially activate *in vivo* and target EBV positive PTLD. However, we recorded no notable reduction in tumour occurrence or time to tumour development (Table 3.3). These findings are consistent with the results reported by Lacerda et al (1996). Notably, three out of twelve animals which received PBMC:LCL ratios higher than 0.5:1 did not develop tumours (table 3.3). It is possible that tumours in these cases were prevented because a larger number of unfractionated PBMC would contain more EBV-specific memory CTLs than lower PBMC doses which could potentially activate and expand in the SCID mouse and control the EBV driven B cell proliferation. Due to limited time available, higher doses of PBMC were not injected, but further investigation with varying doses of unfractionated PBMC are required to clarify the role of PBMC in suppression of tumour outgrowth. Previously it has been shown that high doses (10^7 cells) of PBMC from EBV seropositive donors cause I/P tumours in SCID mice on their own (Lacerda et al, 1996). Therefore it is possible that a higher number of PBMC infused subcutaneously to prevent LCL tumours might themselves result in tumours. In our study we failed to observe PBMC induced S/C tumours in the control mice that received PBMC only. Further experiments would clarify this point.

In the next set of experiments we investigated whether *in vitro* expanded autologous EBV-specific polyclonal CTLs would alter outgrowth of LCL tumours in the SCID model. We titrated the number of CTLs by varying the CTL:LCL ratios injected. The goals were: (1) to observe whether EBV-specific CTLs could control EBV induced proliferation of autologous LCL *in vivo* (2) to determine if a minimum critical CTL to tumour cell ratio was required for effective T cell control of EBV induced B cell proliferation.

Ten T cell lines were generated in this study and all contained CD4 and CD8 positive cells, cell types known to play an important role in immune responses to

viruses and cancers (Greenberg, 1991; Zajac et al, 1998). They were HLA-restricted and EBV-specific as confirmed by the lysis of HLA-matched LCL and the failure of lysis of HLA mismatched LCLs and K562 cells in conventional chromium release assays (Brunner et al, 1968). Polyclonality of CTL lines was preserved by limiting the *in vitro* culture time to 4-6 weeks, because it has been reported that prolonged *in vitro* stimulation of T cells results in preferential selection of a subpopulation directed against one immunodominant epitope and may not reflect the true anti-EBV T cell repertoire of the donor (Dietrich et al, 1997; Faure et al, 1998).

All ten CTL lines prevented outgrowth of autologous LCL tumours in all SCID mice at CTL:LCL ratios as low as 0.5:1 (table 3.5). CTLs also prevented tumours at CTL:LCL ratio of 0.25:1 in 70% cases. Our results are similar to the findings of other groups (Boyle et al, 1993; Buchsbaum et al, 1996) and show that CTLs are important effectors in controlling EBV driven B cell proliferation. Although at lower ratios CTLs failed to control tumour outgrowth, the average time to tumour development was prolonged in all animals which received a mixture of CTL and LCL cells as compared to the LCL only control group. Our results suggest that EBV-specific CTLs can effectively control *in vivo* expansion of EBV infected B cells at very low cell numbers and can prolong survival in cases where tumour outgrowth is not controlled completely.

Since prolonged *in vitro* culture of EBV-specific CTLs results in preferential selection and expansion of CTLs directed against one or two immunodominant epitopes (Dietrich et al, 1997; Faure et al, 1998), in the next set of experiments we investigated if *in vitro* expanded EBV epitope-specific CTL lines would affect outgrowth of autologous LCL tumours *in vivo* in the SCID mouse. Most frequent known HLA restricted CTL responses in EBV infected hosts are to immunodominant epitopes from EBNA 3, 4 and 6 and are associated with a wide range of HLA types (Murray et al, 1992; Khanna et al, 1992; Gavioli et al, 1992). Therefore, we studied the effect of CTL specific for immunodominant epitopes from these 3 latent EBV genes (Table 3.9 and 3.12) in donor 5 and 2. CTL lines specific for lytic cycle antigens BMLF1 and gp350 were also investigated in one

of the two donors (donor 5) because it has been reported that these antigens are often expressed in PTLD tumours and have been detected in biopsy specimens (Rea et al, 1994; Oudejans et al, 1995; Montone et al, 1996; Niedobitek et al, 1997). In our study all peptide-specific CTLs lysed comparable percentages of autologous peptide pulsed PBMC and LCLs *in vitro* (figure 3.4). However, each CTL line showed a higher percentage of lysis for autologous peptide coated targets as compared to autologous LCL cells. Similar results have been reported by Hill et al who also observed reduced *in vitro* killing of autologous LCL cells as compared to autologous peptide coated target cells by peptide-specific CTLs (Hill et al, 1995). The exact cause of this reduced LCL killing has not been addressed previously and requires further investigation.

To investigate if *in vitro* and *in vivo* ability of peptide-specific CTLs to grow and control growth of EBV infected cells was dependant upon the number of CTLp present in the peripheral blood attempts were made to measure CTLp frequencies by LDA for each epitope in PBMC of both donors (2 and 5). The overall detection of CTLp was low in our study and this was thought to be due to the fact that fresh PBMC were not available from the donor and CTLp frequency was measured from frozen PBMC. The test could not be repeated because of the limited number of PBMC available from the donor.

Although we could only detect precursors to the two EBNA6 epitopes in donor 5 PBMC, all six peptide-specific CTL lines gave specific lysis of autologous LCLs *in vitro*. We further investigated whether all the peptide-specific CTL lines would affect the outgrowth of autologous LCL tumours *in vivo* in the SCID model. All peptide-specific CTLs prevented tumour occurrence at a CTL:LCL ratio of 1:1. However, only the CTLs directed against the KEHVIQNAF epitope of EBNA6 prevented autologous LCL induced tumour outgrowth at lower ratios. This CTL line prevented LCL tumours at CTL:LCL ratio as low as 0.06:1. Out of the two EBNA6 epitopes the number of CTL precursors for this epitope was higher than that for the EENLLDFVRF EBNA6 epitope (table 3.10). Results suggest that *in vivo* EBNA6 epitope KEHVIQNAF might be the immunodominant epitope for donor 5. However, it must be noted that all six CTL lines showed comparable

percentage of lysis of autologous LCL *in vitro* and CTL specific for BMLF1, EBNA4 and EENLLDFVRF EBNA6 epitopes prevented tumour outgrowth at CTL:LCL ratio of 1:1 (table 3.11). This raises the possibility that perhaps higher numbers of these peptide-specific CTLs could be successful in controlling growth of EBV infected malignant cells *in vivo*. Due to limited time and number of cells available from the donor, the experiment could not be repeated with higher cell numbers. Further investigations would be required to confirm our results. An interesting study would be to combine more than one epitope-specific CTL line and observe their combined effect on tumour outgrowth.

In another similar experiment we expanded three peptide-specific CTL lines from PBMC of donor 2. EBV peptides used were two EBNA6 epitopes; KEHVIQNAF and EENLLDVFRM and an EBNA3 immunodominant epitope SVRDRLARL (Rickinson & Moss, 1997). CTL precursors for all three epitopes were detected in the peripheral blood of this donor. 100% tumours were prevented at CTL:LCL ratio of 1:1 by all three peptide-specific CTL lines and 66% at CTL:LCL ratio of 0.5:1 (table 3.13). Results show that peptide-specific CTLs on their own were not as effective as polyclonal CTLs in controlling autologous LCL tumour outgrowth because polyclonal CTL from the same donor prevented tumour outgrowth down to CTL:LCL ratio of 0.25:1 in 100% animals (table 3.5). However, it cannot be assumed that polyclonal CTL from this donor were more effective in controlling tumour outgrowth than peptide-specific CTLs because tumour response to CTLs directed against a limited number of EBV epitopes was measured and the immunodominant epitope for the donor was not identified. Immunodominant CTL epitopes could be identified by T cell cloning or prolonged *in vitro* culture of LCL stimulated polyclonal CTLs, which would allow time for selection of the CTL specific for the immunodominant epitope(s) (Dietrich et al, 1997; Faure et al, 1998), which could then be detected by observing the killing of target cells pulsed with different peptides in a cytotoxicity assay.

Although all peptide-specific CTL lines lysed autologous LCLs *in vitro*, overall the majority of epitope-specific CTLs failed to have an appreciable effect on tumour outgrowth *in vivo* at the doses injected. The results suggest that perhaps *in*

vitro and *in vivo* parameters for effective control of EBV immortalized B cells by T cells targeting specific epitopes are different. Compared to polyclonal CTLs a higher number of peptide-specific CTLs may be required for more effective control of EBV driven B cell proliferation *in vivo*.

The results of this pilot study are encouraging because tumour outgrowth was prevented by at least one peptide-specific CTL line from donor 5 at very low cell numbers. This suggests that if an immunodominant epitope is identified for an individual donor it could serve as an effective CTL target for immunotherapy of PTLD. However, identification of an immunodominant epitope for each individual patient would be time consuming and labour intensive. A logical approach would be to use peptide pools representing a set of already defined EBV immune epitopes to expand CTL lines *in vitro* and then observe their effect on proliferation of EBV infected cells *in vivo*. This was a preliminary study and to draw a conclusion that like polyclonal CTLs, peptide-specific CTLs might have a place in immunotherapy of EBV positive tumours, a study on a larger number of individuals and a wider variety of EBV CTL epitopes is required. If subsequent *in vivo* investigations can confirm our results, epitope-specific CTLs could be used in treatment of EBV related malignancies in humans.

Success of adoptive immunotherapy in treatment of PTLD has suggested the possibility of extension of this treatment to other EBV related malignancies. EBV is associated with a subset of HD (Klien et al, 1979; Gutensohn & Cole, 1980; Weiss et al, 1987; 1989) and NPC (Klein et al, 1979; de Thé, 1982), which are more common and of bigger public health concern than PTLD. In HD, EBV is carried in the R/S cells (Weiss et al, 1987) and it has been reported that EBV-specific CTLs can lyse EBV infected R/S cells *in vitro* (Sing et al, 1997). Recently, Roskrow et al have successfully generated EBV-specific CTL from a proportion of EBV-positive HD patients and shown that these cells can persist *in vivo* for more than 13 weeks (Roskrow et al, 1998). Rooney et al treated three of the cases from Roskrow's study with gene-marked autologous CTLs and observed transient improvement in all cases (Rooney et al, 1998). However, the potential of CTL therapy of HD and NPC is complicated by the fact that unlike PTLD, where

cells express all the EBV latent antigens, HD and NPC tumours only express EBNA1, LMP1 and LMP2a and b (Brooks et al, 1992; Busson et al, 1992; Deacon et al, 1993; Rickinson & Kieff, 1996). This reduces the number of CTL epitopes presented on the tumour cell surface and enhances evasion of the immune response.

CTL targets have been recognized within EBNA1 and CD4 positive CTLs against a peptide epitope from EBNA1 have been expanded from healthy donors (Khanna et al, 1995). However, these CTLs failed to lyse infected cells *in vitro*. Levitskaya et al have shown that EBNA1 epitopes are not processed and presented on cell surface via the class I pathway because of inhibition of peptide processing and presentation by a glycine-alanine repeat region within EBNA1 sequence (Levitskaya et al, 1995). Thus CD8 T cells directed against EBNA1 are not generated in a normal immune response and can not be used for immunotherapy. Subdominant epitopes within sequences of LMP1 and LMP2a and b have been identified which are presented in association with a limited number of HLA types (Rickinson & Moss, 1997). This suggests that immunotherapy of HD and NPC would be feasible with CTL directed against epitopes from LMP1 and LMP2a and b. Recently Khanna et al have identified CTL epitopes YLLEMLWRL and YLQQNWWTL, within LMP1 which are recognized by both polyclonal and peptide-specific CTLs (Khanna et al, 1998). They have reported that although the LMP1-specific CTL responses constitute a minor component of the anti-EBV CTL response, the CTL were able to lyse HLA A2 expressing EBV-infected B cells. These findings are encouraging because of the possibility of using CTLs directed against subdominant EBV epitopes to control *in vivo* proliferation of EBV positive HD and NPC.

In our study we tested the effect of *in vitro* expanded CTLs specific for the LMP1 epitope YLLEMLWRL from donor 6 and 7 on autologous LCL tumours in SCID mice. Peptide-specific CTLs from donor 6 prevented tumours at CTL:LCL ratios of 0.25:1 and higher. However, donor 7 CTL failed to alter tumour outgrowth at all CTL:LCL ratios injected (table 3.14). Although we tested LMP1-specific CTLs from only two donors in a small number of animals, suppression of tumour

outgrowth in one of two cases is encouraging since it shows that a CTL response to a subdominant epitope can be successful in controlling EBV induced B cell proliferation *in vivo*. Apart from tumours with a restricted EBV gene expression pattern CTLs directed against subdominant epitopes may also have a place in the treatment of PTLD. Although, most PTLD cases express all the EBV latent antigens, cases have been identified which have a restricted EBV gene expression pattern similar to HD and NPC (Thomas et al, 1990; Gratama et al, 1991; Rea et al, 1994; Delecluse et al, 1995). CTLs targeting these subdominant epitopes might be effective in the immunotherapy of these tumours.

Since *in vitro* expanded CTLs prevented outgrowth of autologous LCL tumours in SCID mice in the next set of experiments we studied the effect of a single dose of *in vitro* expanded autologous polyclonal CTLs on growth of already established subcutaneous LCL tumours in SCID mice and compared intravenous and intratumour routes of CTL delivery. Our results showed that a single fixed dose CTL injection caused reduction in tumour mass in all I/V treated and two I/T treated animals. These findings are similar to those reported by Lacerda et al who showed that *in vitro* activated EBV-specific CD8+ T cells were effective in controlling autologous PBMC or LCL tumours in SCID mice (Lacerda et al, 1996). Intravenous delivery of CTLs was a superior route and resulted in improved survival as compared to the intra-tumour treated and non-treated control groups. Kaplan-Meier survival analysis showed that the probability of survival for intra-tumour treated group became zero at day 40 while it remained at 0.75 for the I/V treated group till the end of the experiment(figure 3.10).

Since a single I/V injection of CTL led to tumour mass reduction in SCID mice, in the next experiment we investigated if multiple fixed dose I/V CTL injections delivered weekly would increase the probability of survival and control growth of tumours of different masses. Our results demonstrated that CTL therapy was successful in causing total tumour regression if initiated at a tumour mass below 500mm^3 (figures 3.11 - 3.13). However, no beneficial effect was observed once tumour mass had increased beyond 2000 mm^3. These findings indicate that multiple CTL injections can cause a rapid reduction in tumour mass, however, as

in *in vitro* experiments, effective lysis of target cells *in vivo* is also dependant upon a critical CTL to tumour cell ratio. Tumours with a large mass at the start of therapy might require a higher dose and/or more frequent injection of CTLs to effectively control their growth.

These were preliminary studies using small numbers of donors and animals, but the results demonstrate that autologous EBV-specific CTLs are effective in improving survival and causing regression of EBV positive tumours in SCID mice. Results suggest that this effect is dependent upon the route of delivery and the initial tumour burden. I/V injection route which is more comparable to human situations (Servida et al, 1993; Rooney et al, 1995) gave better results in terms of overall survival. We were also successful in demonstrating the importance of optimum CTL:tumour cell ratio for effective control of tumour growth. Our findings also show that multiple CTL injections may be more effective than single injections in controlling EBV-driven B cell proliferation. A larger study testing the efficacy of different numbers of CTL on already established tumours is required to further clarify our results. We did not include allogeneic targets in our *in vivo* study, however, failure of the CTLs to lyse HLA mismatched targets *in vitro* constituted evidence of HLA restriction of our CTL lines. In subsequent studies it would be interesting to investigate if CTLs would lyse allogeneic cells *in vivo* as well as *in vitro*.

It has been reported that CTLs infused into SCID mice successfully home on to autologous tumour cells *in vivo* (Greenberg et el, 1988; Alexander & Rosenberg, 1991; Wallace et al, 1993). However, detection of CD3 positive cells in tumour tissue is not sufficient evidence of their cytotoxicity *in vivo*. It has been suggested that *in vivo* CTLs cause tumour regression by both direct cytotoxicity and by production of IFN-γ (Barth et al, 1991). We did not investigate IFN-γ production by the infiltrating T cells in our LCL tumours treated with autologous CTLs, however, in future studies this issue could be addressed by immunohistochemical double staining of tumour sections for CD3 and IFN-γ expression.

In conclusion, the overall results of our study on immunotherapy of EBV related PTLD are consistent with earlier investigations both in humans and SCID mice

(Rooney et al, 1995; Lacerda et al, 1996). We have taken the investigations further by titrating the CTL numbers and showing that effective control of EBV driven B cell proliferation is dependant upon a minimum CTL:LCL ratio (.25:1 in our experiments), below which CTLs fail to effect tumour outgrowth. Our results also suggest that peptide-specific CTLs targetting EBV immunogenic epitopes could have a place in immunotherapy of EBV related tumours, especially of tumours which have a restricted EBV gene expression pattern and cannot be treated by polyclonal CTLs. Future studies on a larger panel of donors and a larger group of animals would be required to confirm our results and assess the true potential of peptide based CTL therapy of EBV related diseases.

5.2 In situ Hybridization

Many *in vitro* studies have identified cytokines as soluble autocrine or paracrine growth factors for EBV immortalized cells (Randhawa et al, 1994; Oppenheim & Fugiwara, 1996; Su et al, 1997; Rochford et al, 1997; Sairenji et al, 1998). It has been suggested that cytokines maintain the sustained growth of EBV positive cells both *in vitro* and *in vivo* (Tosato, 1987; Straus et al, 1993; Randhawa et al, 1997). Most previous studies have investigated cytokine profiles of EBV related diseases with RT-PCR (Nalesnik et al, 1999), a technique which is sensitive but does not localize the cytokine to the cell type producing it. In our study we used non-isotopic in situ hybridization (NISH) to study the cytokine expression pattern of ten human PBMC-induced I/P tumours in SCID mice (*in vivo* model for PTLD), a tonsil from an acute IM patient, thirteen PTLD, six NPC and five HD tumours. We focused on detection of IL-2, 4, 6, 10 and IFN-γ, cytokines reported to regulate growth and proliferation of EBV infected cells (Tosato et al, 1990; Estrov et al, 1993; Miyazaki et al, 1993; Durandy, et al, 1994; Tosato et al, 1998; Takashi & Puri, 1997). NISH technique was performed on paraffin wax embedded tissue to histologically localise the cytokine mRNA signal to the cell type responsible for its production. Subsequent staining of serial sections for cell surface markers was undertaken to identify the predominant cell type encoding cytokine transcripts within the tissues tested.

5.2.1 Standardization of NISH Technique

We used DIG labelled probes in our study because DIG is a plant alkaloid, is not present in mammalian tissue, and has previously been used successfully for nucleic acid detection by NISH (Herrington et al., 1989). Although previous studies have reported that radioisotopic labels are more sensitive than non-radioactive reporter molecules (Li et al, 1993; Stefaneanu et al, 1993), we selected a non-isotopic label since signal localisation is reported to be more accurate. This is because, unlike radioisotopically labelled sections non-isotopically probed tissue does not require to be layered in emulsion, and the signal is localised in the tissue itself rather than in the emulsion layer (Wiethege et al, 1991). Non-isotopic labels are also more convenient to use than radioactive probes in large ISH studies

because they are safer and there is no requirement for a designated work area (Hermanson et al, 1994). Non-radioactive reporter molecules do not have a half life and therefore do not degrade like radioisotopic labels and do not require a prolonged development time (Hermanson et al, 1994).

In our study the NISH technique was standardised on cytospin preparations of formalin-fixed human PHA-stimulated PBMC and paraffin-wax embedded sections of normal human tissue. Formalin was preferred to ethanol as a fixative for cytospin preparations because ethanol is less effective in immobilizing RNA and can cause loss of target during tissue processing (Lawrence & Singer, 1985). Although cryopreservation of tissue has been reported to be better for probe penetration (Le Guellec et al, 1992), formalin fixed and paraffin-wax embedded tissue was used because it was the only material available from our test samples. We used PHA blasts as positive controls for cytokine detection because a consistently strong mRNA signal could be detected for all the cytokines on our panel in these cells with each NISH run. Normal human lymphoid tissue was not routinely used as a control because mRNA transcripts could not be consistently detected in this tissue. The sensitivity of mRNA detection by NISH was tested by inclusion of a probe for detection of β-actin mRNA. Sections that gave a low signal for β-actin also gave a poor signal for all the cytokines. This was attributed to either poor preservation of tissue resulting in mRNA degradation (Bromely et al., 1994) or due to tumour necrosis, as observed by H&E staining of the sections. H&E staining of sections from some of the tumours showed large necrotic areas and in serial sections the cells positive for β-actin and cytokine transcripts were interspersed among areas of necrotic tissue. These tumours also showed a reduced number of cells positive for EBERs mRNA and cell surface markers. The results from these tumours could not interpreted.

Negative controls included no-probe sections to exclude non-specific binding by the multiple layers of antibodies. RNase-treated sections were included as target removal controls, which remained negative because of digestion of mRNA prior to application of the probe. The specificity of the technique was tested by

inclusion of non-human tissue (mouse tissue), which lacked the target sequences. No signal was detected in any of the negative controls tested during the study.

Probe access to mRNA target was facilitated by dewaxing and dehydration of sections followed by Pk digestion. Digestion with Pk was essential because fixation of tissue with formalin causes cross-linking of nuclear proteins and nucleic acids (Feldman, 1973). Pk treatment broke down these cross-links and made the target more accessible (Hopman et al, 1989). The conditions of Pk digestion were optimised for both paraffin-wax embedded and formalin-fixed PHA-stimulated blasts. Optimization of Pk concentration and time and temperature of digestion were critical for penetration of the probe into the tissue and preservation of tissue morphology. We observed that both over or under digestion of the tissue resulted in weak or no mRNA signal. Over digestion often resulted in loss of tissue morphology and detachment of sections from slides during the procedure.

Our initial attempts to detect and amplify cytokine mRNA signal in both cytospin preparations and paraffin embedded tissue failed. Sensitivity of the technique was increased by introduction of FITC as an additional reporter molecule in the protocol. Addition of extra antibody layers could have potentially resulted in non-specific signal but this was avoided by including additional incubation of sections in normal sheep, rabbit and donkey sera (host animals for primary, secondary and tertiary antibodies) after stringency washes. The streptavidin-biotin system was chosen for signal amplification because streptavidin has a high affinity for biotin and each streptavidin molecule contains four biotin-binding sites, which gives a high amplification of the signal (Hoefakker et al, 1995). The biotin in the Dako ABC kit is coupled with alkaline phosphatase (AP) molecules and binding of the AP-biotin complex to four streptavidin sites increases the number of molecules of enzyme available to degrade the substrate. BCIP/NBT was the AP substrate of choice for our study because it reacts for a longer time with AP than most other AP substrates (Cremers et al, 1987; Ehrlein et al, 1994). It has good localisation properties, sensitivity and stability (De Jong et al, 1985; Scopsi & Larson, 1986) and produces a stable purple-blue colour when hydrolysed and reduced by AP.

Solvents do not degrade it and sections can be mounted permanently without loss of signal.

5.2.2 Cytokine Profile of EBV Related Diseases

In our study we analysed the In situ expression of IL-2, 4, 6, 10 and IFN-γ in EBV related diseases, identified the cell type responsible for cytokine production and its association with EBV infection. We investigated EBV positive PTLD tumours, tumours from SCID mice induced by I/P injection of PBMC from EBV seropositive donors, NPC tumours and HD tumours. The cytokine profile of a tonsil from an acute IM patient was also studied to compare the cytokine expression pattern of primary EBV infection with that of EBV related malignancies. This was included because recently Setsuda et al have reported elevated level of cytokine gene expression by lymphoid tissue from acute IM patients as compared to PTLD tumours (Setsuda et al, 1999).

Expression of IL-2, 4, 6, 10 and IFN-γ mRNA was detected in human PBMC induced I/P tumours from SCID mice and cytokine positive areas predominantly equated with areas positive for CD20 and EBERs signal confirming autocrine cytokine production by EBV infected tumour cells. PTLD tumour B cells also expressed a similar cytokine mRNA expression pattern. The tumours were positive for signal of all the cytokines in our panel, however, as compared to IL-2, 4 and IFN-γ, a higher percentage of cells were found to be positive for IL-6 and IL-10, which are both B cell growth factors (Yokoi et al, 1990; Emelie et al, 1992). Our results are different from those of Nalesnik et al who failed to detect any IL-2 and IFN-γ by RT-PCR in 11 PTLD cases (Nalesnik et al, 1999). In our study we detected transcripts of IL-2 and IFN-γ in all the tumours, but the number of cells positive for these cytokines was lower as compared to IL-6 and IL-10. IL-10 is known to suppress IL-2 and IFNγ expression, which may account for the reduced expression of these cytokines in our tumour panel. Our results showed that cytokine mRNA expression was predominantly localized to EBV positive tumour cells suggesting that EBV positive PTLD tumour cells produce cytokines in an autocrine fashion. This might enable them to directly stimulate their own

growth and suppress the anti-EBV cellular immune response by inducing expression of the immunosuppressive cytokine IL-10. It has been reported that PTLD tumours are infiltrated with CD4 positive cells in their early stages (Perera et al, 1998) and these T cells might contribute to the pathogenesis of the tumour by providing cytokines for initial cell growth. However, immunohistochemical analysis of four of the PTLD tumours in our study showed less than 1% infiltrating CD3 positive T cells, interspersed among CD20 and EBER positive cells. It is likely that these infiltrating T cells may also be expressing cytokines but because of their sparse distribution it was not possible to localize cytokine production to these cells on serial sections. Double and triple labeling techniques for cytokine and EBERs mRNA detection and cell surface markers were attempted by using protocols described in a previous study (Rex et al, 1997), but did not give satisfactory results in the time available. If these techniques could be developed and refined, more conclusive results could be obtained about the contribution of infiltrating T cells to pathogenesis of PTLD.

More than 50% cases of HD in Europe are now known to be EBV positive, with the giant R/S and Hodgkin cells carrying the virus (Weiss et al, 1989, Alexander et al, 2000). It has been suggested that EBV might be a co-factor in HD pathogenesis and one possible mechanism of its contribution to tumour pathology and immune evasion might be through induction of cytokine expression. In our study the cytokine and EBERs mRNA signal was predominantly confined to giant CD30 positive R/S cells of which, over 50% were positive for cytokine and EBERs mRNA in all cases. In a recent immunohistochemical study Dukers et al have reported expression of IL-2, 4, 10 and IFNγ by both malignant and infiltrating reactive cells in HD (Dukers et al, 2000). However, we failed to detect cytokine expression or EBERs signal in the infiltrating cells. Our results suggest that in HD cytokine gene expression is limited to EBV positive R/S cells and the virus might regulate tumour proliferation via induction of autocrine cytokine production. We did not investigate cytokine expression pattern of EBV negative HD but Dukers et al found no difference in level of expression of IL-2, 4 and IFNγ between EBV positive and negative HD. However, EBV negative tumours expressed lower levels of IL-10 protein as compared to EBV positive cases

(Dukers et al, 2000). IL-10 is an autocrine B cell growth factor and can also modulate the host anti-tumour immune response by suppression of production of other immuneregulatory cytokines such as IL-12 and IFNγ (Chouaib et al, 1997). In our study three out of five tumours expressed IL-10 mRNA. However due to poor tissue quality we failed to observe a relationship between expression levels of IL-10 and other cytokines on our panel. Further investigation is required on a larger number of both EBV negative and positive HD tumours to confirm our findings.

Previous studies using RT-PCR and intracellular cytokine staining by immunoflowcytometry have reported expression of IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, INF-γ, TNF-α and TGF-β in both NPC tumour cells and the infiltrating T cells (Busson et al, 1987; Huang et al, 1999). Other studies on immunohistochemical detection of individual cytokines in NPC tumours have shown expression of IL-10 and IFNγ (Tang et al, 1999, Fujieda et al, 1999) and IFN-γ signal was predominantly observed in the infiltrating T lymphocytes (Tang et al, 1999). We investigated the cytokine expression pattern of EBV related NPC and the cell type(s) responsible for their production. Our results showed that both the epithelial tumour cells and the infiltrating CD3 positive T cells expressed cytokine transcripts with the predominant expression in the infiltrating cells. We also found that the signal for IL-6 and IL-10 was present in a higher number of both tumour and infiltrating cells as compared to other cytokines. Our findings suggest that in NPC tumour growth is regulated by both autocrine and paracrine cytokine production by the malignant and the infiltrating cells. Since in NPC the infiltrating T cells fail to control the EBV driven malignant cell proliferation our results suggest that paracrine cytokine production by infiltrating cells might contribute positively to the expansion of the NPC tumour. To further investigate our results a study on a larger number of NPC tumours is required and compared with cytokine mRNA expression by EBV negative NPC cases and normal nasopharyngeal tissue.

To observe if the cytokine expression pattern of normal lymphoid cells during acute EBV infection would resemble that of the EBV related malignancies,

tonsillar sections from a case of acute IM were probed for IL-2, 4, 6, 10 and IFNγ mRNA. The IM tonsil tested positive for all the cytokines in our panel with the highest percentage of cells positive for IL-6. Areas of cytokine expressing cells equated with areas positive for EBERs signal in serial sections. Previously, it has been reported that serum levels of IL-6 and IL-10 are raised in patients with acute IM as compared to healthy individuals (Wright-Browne et al, 1998; Tage et al, 1995). In contrast to our study the findings of an immunohistochemical study by Setsuda et al, who reported higher levels of IFNγ production in IM than PTLD tumours (Setsuda et al, 1999). We only detected IFNγ in <1% cells in the IM tonsil. In our study the cytokine expression pattern of the IM tonsil was similar to that of EBV related malignancies. These results suggest that like in EBV positive tumours cytokine transcription by EBV infected B cells in IM may modulate the pathogenesis of the disease.

Overall our results show that EBV infected cells express cytokines in an autocrine fashion. This suggests that EBV infected cells might modulate their growth and regulate the host immune system via the expression of both B cell growth promoting and immunosuppressive cytokine. Our findings and previously reported data suggest that EBV induced B cell immortalisation and proliferation and immune escape are controlled by the virus via the cytokine network. Further studies are required to identify the mechanism by which EBV induces and controls cytokine expression in infected cells. Better understanding of the interaction between EBV and the cytokine network could lead to new immunotherapies for treatment and prevention of EBV related malignancies.

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7. Appendix: Publication

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Essential role for T cells in human B-cell lymphoproliferative disease development in severe combined immunodeficient mice

I. JOHANNESSEN, M. ASGHAR AND D. H. CRAWFORD *Department of Medical Microbiology, The University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, UK*

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Summary. Epstein–Barr virus (EBV)-positive B-cell lymphoproliferative disease (BLPD)-like lesions develop in severe combined immunodeficient (SCID) mice inoculated with peripheral blood mononuclear cells (PBMCs) from EBV-seropositive donors. We used this model to investigate the pathogenesis of EBV-associated BLPD. Tumour incidence fell from 81% to 11% when only B cells were inoculated, suggesting a key role for T cells in tumour formation. This was further underlined by the reduction in tumour incidence from 76% to 7% when PBMCs were depleted of CD4 positive (+ve) helper T cells. Tumour outgrowth was also reduced when PBMC were depleted of CD8 +ve, CD45RA +ve or CD45RO +ve T cells. The majority of PBMC-derived tumours analysed by reverse transcriptase–polymerase chain reaction (RT–PCR) expressed mRNA for interleukin (IL) 2, 4, 6, 10 and interferon (IFN) γ. This is the cytokine pattern seen in activated T cells and includes B-cell growth factors. *In situ* hybridization studies confirmed that the tumour cells themselves express the growth factors, which is consistent with autocrine-stimulated tumour growth.

Our results suggest the following sequence of events: (1) T cells are essential for the initial outgrowth of tumorigenic EBV +ve B cells *in vivo*; (2) the neoplasm sustains its growth in an autocrine, cytokine-stimulated manner; and (3) established tumours become independent of T-cell help.

Keywords: EBV, lymphoma, T cells, cytokines, SCID mice.

INTRODUCTION

The term B-cell lymphoproliferative disease (BLPD) denotes a spectrum of B-cell proliferations arising in 1–10% of organ transplant recipients (Thomas *et al.* 1995). BLPD ranges from a diffuse (viral lymphadenitis-like) polymorphic B-cell proliferation with polyclonal immunoglobulin (Ig) expression to a malignant monoclonal lymphoma. Epstein–Barr virus (EBV), a human herpes virus, is aetiologically associated with BLPD (Crawford *et al.* 1980). Tumour cells generally express the nine EBV latent genes, together with B-cell activation markers and cellular adhesion molecules (CAMs) (Young *et al.* 1989; Thomas *et al.* 1990). This phenotype is similar to that of continually proliferating B lymphoblastoid cell lines (BLCLs) established *in vitro* by infection of B cells with EBV.

Correspondence: Prof D. H. Crawford, Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, UK. E-mail: d.crawford@ed.ac.uk
This work was carried out at the London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK.

The precise mechanism of BLPD development in transplant recipients is yet to be elucidated, although major risk factors include high-dose immunosuppression and primary EBV infection in previously seronegative transplant recipients (Ho *et al.* 1985; Thomas *et al.* 1990). The tumour commonly arises in the brain, gut or transplanted organ, where the microenvironment is thought to provide the right milieu for EBV-driven B-cell proliferation. There is commonly a large non-neoplastic component in BLPD mainly consisting of mature CD4 +ve (helper) CD45RO +ve (memory) T cells (Thomas *et al.* 1990; Perera *et al.* 1998), which may provide a source of soluble growth factors and facilitate tumour growth.

The severe combined immunodeficient (SCID) mouse, which lacks functional B and T cells, is a recent small-animal model for EBV-associated BLPD by virtue of its ready acceptance of human (hu) xenografts (Bosma *et al.* 1983; Mosier *et al.* 1988). Peripheral blood mononuclear cells (PBMCs) from EBV-seropositive healthy blood donors give rise to EBV +ve BLPD in a proportion of animals (hu-PBMC-SCID) (Picchio *et al.* 1992). These SCID tumours are immunoblastic lymphomas (Mosier *et al.* 1990; Purtilo

et al, 1991) closely resembling BLPD in graft recipients (Rowe *et al*, 1991). The tumours are of human B-cell origin and show a surface phenotype identical to that seen in BLCLs and BLPD with expression of all the latent viral proteins, B-cell activation antigens and CAMs (Mosier *et al*, 1990; Rowe *et al*, 1991). BLCLs give rise to similar tumours in the SCID mouse model (Rowe *et al*, 1991).

The present study was designed to investigate the requirements for tumour outgrowth in PBMC-inoculated SCID mice as a model for BLPD in transplant recipients. Tumour incidence was dramatically reduced when CD2 +ve or CD4 +ve cell populations were depleted from PBMCs prior to inoculation. Tumour incidence was reduced to a lesser extent by removal of CD8 +ve, CD30 +ve, CD45RA +ve or CD45RO +ve T cells. Using reverse transcriptase–polymerase chain reaction (RT–PCR), the presence of amplifiable human interleukin (IL) 2, 4, 6, 10, and interferon (IFN) γ, mRNA was regularly detected in the tumour material. *In situ* hybridization studies demonstrated that the transcripts are produced by the tumour cells.

The results suggest that initial tumour outgrowth requires T-cell help but that established tumours are maintained in an autocrine, cytokine-stimulated fashion.

MATERIALS AND METHODS

SCID mice. All animal work was carried out in full compliance with the Animals (Scientific Procedures) Act 1986. C.B17 SCID mice, aged betweeen 3 and 34 weeks, were supplied from a breeding colony kept under specific pathogen-free conditions at the London School of Hygiene & Tropical Medicine.

Blood donors. Aliquots containing 50–100 ml of heparinized blood were drawn from healthy volunteers. Blood bags containing PBMCs were obtained from the National Blood Transfusion Centre in Colindale, London, UK.

EBV serology. Analysis of anti-VCA IgG antibody titre was carried out by routine indirect immunofluorescence (Henle & Henle, 1966).

Separation of PBMC from heparinized whole blood. Whole blood, or buffy coat, was layered over Ficoll-Hypaque (Pharmacia, Sweden) and spun at 540 *g* for 20 min. Plasma was harvested and stored at −20°C for serological analysis. The interface containing the PBMCs was collected and washed twice. Cells were either used directly, or frozen viably and stored in liquid nitrogen.

Fractionation of PBMC by E-rosette separation. PBMCs were fractionated by E-rosette separation by the standard method (Kaplan & Clark, 1974). E-rosette-negative (CD2–ve) cells enriched for B cells, monocytes and natural killer (NK) cells, were harvested, washed, and either used directly or stored at 4°C for later use. E-rosette-positive T cells and NK cells, were briefly resuspended in 1 ml of sterile injectable water in order to lyse sheep red blood cells. The lysis was halted by the immediate addition of a large volume of wash medium. Cells were washed and either used directly or subjected to further *in vitro* manipulation. The enrichment of the E-negative and E-positive populations was determined in separate experiments to be ≥ 97%. Control experiments entailed the injection of SCID mice with unfractionated PBMCs. Whenever possible, the control animals were inoculated at the same time as the test mice.

Cell fractionation using antibody-coated magnetic beads. Monoclonal antibodies (mAbs) to human CD4 (QS4120), CD8 (UCHT4) and CD45RO (UCHL1) were kindly provided by Professor P. C. L. Beverley (The Edward Jenner Institute for Vaccine Research, Compton, Berkshire, UK). mAbs to human CD45RA (SN130) were provided by Professor G. Janossy (Royal Free Hospital Medical School, London, UK). mAb to human CD30 was supplied by Cymbus Biotechnology (Hampshire, UK). The T-cell enriched population was resuspended in 1 ml of culture medium (1 x RPMI−1640, 10% v/v fetal calf serum, 100IU/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l L-glutamine) containing a working dilution of azide-free murine IgG mAbs (approx. 10 μg) to either human CD4 (Reinherz *et al*, 1979a, b), CD8 (Reinherz *et al*, 1980), CD30 (Schwab *et al*, 1982; Schwarting *et al*, 1989), CD45RA (Akbar *et al*, 1988; Serra *et al*, 1988), or CD45RO (Akbar *et al*, 1988; Merkenschlager *et al*, 1988) surface antigens, and was then incubated at 4°C for 30 min. After two washes the cells were incubated with (10 μl/10^6 cells) prewashed sheep anti-mouse IgG-coated magnetic beads for 10 min at 4°C. The uncoated cells were collected by a 3 min application of a magnetic particle separator (Vartdal *et al*, 1987). Successful depletion of a cell subpopulation was determined in separate experiments to be > 85%, except for the CD30-depleted population which was > 70% depleted.

Lectin-stimulation of PBMC. 10 μg/ml phytohaemagglutinin (PHA) was added to PBMCs at 2×10^6 cells/ml (Goldstein and Hayes, 1978). Cells were cultured for 24 h at 37°C in 5% CO_2 and washed before *in vivo* use.

Inoculation and monitoring of SCID mice. Cells were injected intraperitoneally (ip) in 500 μl of culture medium. Between 42×10^6 and 54×10^6 unfractionated PBMCs were inoculated into each control animal. This number of cells was also either PHA-treated *in vitro* for 24 h and inoculated into each test mouse, or separated into E-negative and E-positive cells followed by depletion of a specific T-cell subpopulation and inoculation of the recombined E-negative and subpopulation-depleted E-positive cells into each test mouse. Each test group consisted of three animals. On showing signs of illness, or after a preset time limit of 100 d, mice were culled and lung, liver, spleen, and any tumour tissue were removed. Pieces from each tissue were fixed in neutral buffered formalin and snap-frozen.

Classification of blood donors. We and colleagues have previously found PBMCs from individual blood donors to give rise to tumours in a similar proportion of inoculated SCID mice upon repeat testing (Picchio *et al*, 1992; Johannessen *et al*, 1998). Thus our panel of 24 blood donors contained 8 high (> 70% tumour incidence), 12 intermediate (30–70% tumour incidence), 2 low (1–30% tumour incidence), and 2 no incidence donors.

Immunohistochemistry. All tissues were routinely stained with haematoxylin and eosin (H&E). Frozen or paraffin wax-embedded tissue sections were immunostained with a panel of primary antibodies: polyclonal anti-EBNA1–6, anti-CD3 mAb (Dako, Buckinghamshire, UK); anti-CD4 mAb

Table IA. Primer sequences used for RT-PCR amplification of β-actin and human cytokine gene transcripts (IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ).

Human gene/primers	Primer sequences (5′→3′)	Product size (bp)‡
β-actin*		
5′:	5′ GTG GGG CGC CCC AGG CAC CA 3′	540
3′:	5′ CTC CTT AAT GTC ACG CAC GAT TTC 3′	
IL-2*		
5′:	5′ ACT CAC CAG GAT GCT CAC AT 3′	266
3′:	5′ AGG TAA TCC ATC TGT TCA GA 3′	
IL-4†		
5′:	5′ CGG CAA CTT TGA CCA CGG ACA CAA GTG CGA TA 3′	344
3′:	5′ ACG TAC TCT GGT TGG CTT CCT TCA CAG GAC AG 3′	
IL-5*		
5′:	5′ ATG AGG ATG CTT CTG CAT TTG 3′	405
3′:	5′ TCA ACT TTC TAT TAT CCA CTC GGT GTT CAT TAC 3′	
IL-6*		
5′:	5′ ATG TAG CCG CCC CAC ACA GA 3′	190
3′:	5′ CAT CCA TCT TTT TCA GCC AT 3′	
IL-10†		
5′:	5′ AAG CTG AGA ACC AAG ACC CAG ACA TCA AGG CG 3′	328
3′:	5′ AGC TAT CCC AGA GCC CCA GAT CCG ATT TTG G 3′	
IFN-γ*		
5′:	5′ AGT TAT ATC TTG GCT TTT CA 3′	356
3′:	5′ ACC GAA TAA TTA GTC AGC TT 3′	

*Yamamura *et al* (1991, 1992).
†Clontech Laboratories, USA.
‡bp, base pairs.

(Dako); anti-CD8 mAb (Dako); anti-CD20 mAb (Dako); anti-CD45RB mAb (Dako); anti-mouse MHC1D^{d} mAb (Serotec, Oxford, UK). Bound antibody was visualized with either a peroxidase [peroxidase antiperoxidase (PAP) test] or alkaline phosphatase label [alkaline phosphatase antialkaline phosphatase (APAAP) test] in accordance with standard methods (van Noorden, 1986). EBNA staining was detected by a routine indirect anti-complement technique (Reedman & Klein, 1973). Cells were quantified by counting the numbers of labelled and unlabelled cells in five high-power fields (× 1000). The results were expressed as a percentage of marker-positive cells in the total cell population counted.

Reverse-transcriptase–polymerase chain reaction (RT-PCR). RT-PCR was carried out essentially as described by Hart *et al* (1988). Total RNA was extracted from control cell pellets or tumour tissues using RNAzol^{TM}B in accordance with the manufacturer's protocol (Cinna/Tel Test, TX, USA). The synthesis of complementary DNA (cDNA) was carried out using an 'RT-PCR Kit' from Stratagene Cloning Systems (CA, USA) in accordance with the manufacturer's protocol using reverse transcription from random primers by reverse transcriptase. PCR was carried out using sequence-specific primers synthesized by Oswel DNA Services (Hampshire, UK) (see Table IA). The IL-10 primers were not found to cross-amplify EBV-vIL-10. Positive and negative control samples, as well as sterile distilled water (SDW) as a DNA template-free negative control, were included in each run. Human β-actin served as a control to ensure the presence of amplifiable cDNA in the samples. The PCR products were run on a 2·5% w/v NuSieve 3:1 agarose (FMC BioProducts, ME, USA) gel and blotted onto Hybond N + nylon membranes (Amersham International, Buckinghamshire, UK) in accordance with the method of Southern (1975) (for probes, see Table IB). The RT-PCR was sensitive enough to detect $10^{2}-10^{3}$ copies of each of the IL-2, IL-5, IL-6, and IFN-γ mRNAs, or approx. 25 attomoles of each of the IL-4 and IL-10 mRNAs (Clontech, USA).

In situ hybridization (ISH) for EBV-encoded small RNAs (EBERs). ISH for EBERs was carried out on paraffin wax-embedded tissue sections by routine methods (Howe & Steitz, 1986).

In situ hybridization (ISH) for cytokine mRNA. ISH was carried out essentially as described by Fleming *et al* (1992) and Karr *et al* (1995), using digoxigenin-labelled oligonucleotide probes supplied by R & D Systems, UK. Positive signals were amplified with sheep anti-digoxigenin antibody (Boehringer Mannheim, UK), followed by a biotin-labelled anti-sheep antibody (Vector, UK). Bound antibody was detected using streptavidin–biotin/alkaline phosphatase (ABC) complex kit (Dako), and the complex was visualized with a 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) substrate (Sigma, UK). Positive controls included PHA-stimulated PBMCs, and negative controls included unprobed sections and RNAase-treated

Table IB. Probe sequences used in RT-PCR to detect β-actin and human cytokine gene transcripts (IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ).

Human gene	Probe sequence (5′→3′)
β-Actin	5′ GGA TAG CAA CGT ACA TGG CT 3′
IL-2	5′ CCC TGG GTC TTA AGT GAA AG 3′
IL-4	5′ GTC TGT TAC GGT CAA CTC GG 3′
IL-5*	5′ GCC AAT GAG ACT CTG AGG ATT CCT G 3′
IL-6*	5′ TCT TGT TAC ATG TCT CCT TTC TCA G 3′
IL-10*	5′ CAG GTG AAG AAT GCC TTT AAT AAG CTC CAA GAG AAA GGC ATC TAC AAA GCC ATG AGT GAG TTT GAC ATC 3′
IFN-γ	5′ GCT ACA TCT GAA TGA CCT GC 3′

* Yamamura *et al* (1991,1992).

sections. These controls were included in every staining run and results were used only if control staining was satisfactory.

Statistical analysis. The statistical tests used in this study were the chi-squared (χ^2) test with Yates' correction, the Fisher's exact test and the Wilcoxon rank sum test.

RESULTS

Immunohistology of PBMC-derived tumours

The analysis of a total of 27 PBMC-derived peritoneal tumours consistently showed the lesions to comprise of large EBV +ve immunoblasts when stained with H & E, EBNA and/or EBERs. H&E further showed areas of necrosis. Detailed analysis of 12 of the tumours demonstrated a human CD45RB +ve, human CD20 +ve, mouse MHC1D^d-negative phenotype, indicating that they were human B-cell lesions. Tumours contained < 6·6% human CD3 +ve T cells, the majority of which were CD8 +ve T cells with a small CD4 +ve population.

Characterization of cell type required for tumour outgrowth in SCID mice

Initially, the separation procedure was tested to determine the enrichment of separated populations and whether cell manipulation and/or cell loss during *in vitro* manipulations significantly affected the tumour incidence. 25×10^7 PBMCs from each of two high incidence donors were separated into E-positive and E-negative cells and recombined, prior to inoculation in equal numbers into each of five animals. Six out of 10 (60%) mice inoculated with recombined E-positive and E-negative cells developed tumours, whereas 19 out of 22 (86%) of the controls developed tumours. Although there was some loss of tumour outgrowth, this was not a significant difference (Fisher's exact test: $P > 0{\cdot}1$). A comparison of the time to tumour did not show a significant difference (Wilcoxon rank sum test: $P > 0{\cdot}1$). The results are shown in Table II. In light of these results, it was concluded that *in vitro* manipulations did not significantly affect tumour incidence in SCID mice.

In order to characterize the cell type(s) required for tumour development in SCID mice, 15×10^7 PBMCs from each of three high incidence donors were separated by E-rosetting and the E-negative and E-positive cells inoculated separately ip into two groups of three test mice each. One out of nine (11%) mice inoculated with either E-negative or E-positive cells developed tumour. In comparison with the earlier results, 21 out of 26 (81%) PBMC-inoculated control mice developed tumours. The results are shown in Table II. This statistically significant reduction in tumour incidence observed in mice injected with B and T cells alone (Fisher's exact test, $P < 0{\cdot}001$) suggests that despite their B-cell origin, both B and T cells are required for tumour outgrowth. The possibility that T-cell-derived soluble factors are required for tumour outgrowth was therefore investigated.

Analysis of the role of T-cell activation in tumour induction

PBMCs ($13 \times 10^7 - 16 \times 10^7$) from each of 10 EBV-seropositive donors were PHA-treated for 24 h *in vitro* prior to ip inoculation of equal cell numbers into three test mice for each PBMC donor. Cells from two high, five intermediate, one low, and two no incidence donors were used. Tumours occurred in 18 out of 30 (60%) mice inoculated with PHA-stimulated PBMCs, whereas 27 out of 49 (55%) control mice developed tumours. Statistical analyses showed that prior *in vitro* PHA treatment of PBMCs did not significantly affect the tumour incidence (chi-squared test with Yates' correction: $P > 0{\cdot}8$). However, the time from inoculation to tumour development was significantly shortened with PHA treatment in high and intermediate incidence donors (Wilcoxon rank sum test: $P < 0{\cdot}05$). The results are shown in Table III.

Furthermore, when between 15×10^7 and 16×10^7 PBMCs from each of three high and one intermediate incidence donors were depleted of CD30 +ve-activated T cells prior to inoculation into groups of three test mice, 3 out of 12 (25%) test mice developed tumours, whereas 29 out of 36 (81%) control mice developed lesions (Table II) (Fisher's exact test, $P < 0{\cdot}001$). The median time to tumour was significantly increased in the test mice compared with the control animals (Wilcoxon rank sum test: $P < 0{\cdot}05$).

These data suggested the importance of activated T cells in tumour formation, and therefore experiments were

Table II. Data from cell separation and cell depletion experiments.

Procedure	Cells injected	Number of cells injected per mouse ($\times 10^6$)	Number of tumours/ number of mice injected (%)	Median time to tumour (days)
Injection of recombined E– and E+ cells	PBMC	49–54	19/22 (86)	61
	E– and E+ cells	37–42	6/10 (60)	58
Injection of either E– or E+ cells alone	PBMC	44–54	21/26 (81)	55
	E– cells	9–11	1/9* (11)	70
	E+ cells	19–26	1/9* (11)	86
Injection of E– cells recombined with E+ cells depleted of a particular T-cell subset	PBMC	42–54	22/29 (76)	56
	E– and CD4-depleted E+ cells	10–22	1/15† (7)	98
	PBMC	44–54	22/29 (76)	58
	E– and CD8-depleted E+ cells	18–31	7/18‡ (39)	60
	PBMC	44–54	29/36 (81)	58
	E– and CD30-depleted E+ cells	11–38	3/12§ (25)	71¶
	PBMC	49–54	19/24 (79)	55
	E– and CD45RA-depleted E+ cells	8–35	6/15** (40)	64
	PBMC	49–54	17/21 (81)	55
	E– and CD45RO-depleted E+ cells	11–26	3/12†† (25)	61

Significance compared with the relevant unfractionated PBMC controls (shown in the table): *$P < 0{\cdot}001$. †$P < 0{\cdot}001$. ‡$P < 0{\cdot}05$. §$P < 0{\cdot}001$. ¶$P < 0{\cdot}05$. **$P < 0{\cdot}05$. ††$P < 0{\cdot}005$.

undertaken to determine the T-cell subpopulation(s) required.

Characterization of T-cell subpopulations required for tumour outgrowth.

E-rosette-positive cells separated from 13×10^7 to 16×10^7 PBMCs from each of seven high and six intermediate incidence donors were depleted of either CD4 +ve, CD8 +ve, CD45RA +ve or CD45RO +ve T cells prior to inoculation into groups of three test mice. Tumours arose in 1 out of 15 (7%) mice receiving CD4-depleted PBMCs, whereas 22 out of 29 (76%) control animals developed lesions (chi-squared with Yates' correction, $P < 0{\cdot}001$). Tumours developed in 7 out of 18 (39%) mice injected with CD8-depleted PBMCs, whereas tumours arose in 22 out of 29 (76%) controls (chi-squared with Yates' correction:

Table III. Data from activation experiments using phytohaemagglutinin (PHA).

Donor subgroup/ cells inoculated		Number of tumours/ number of mice (%)	Median time to tumour (days)
High incidence donors	Untreated PBMC	18/23 (78)	58
	PHA-treated PBMC	6/6 (100)	51*
Intermediate incidence donors	Untreated PBMC	8/15 (53)	62
	PHA-treated PBMC	9/15 (60)	50*
Low incidence donors	Untreated PBMC	1/11 (9)	46
	PHA-treated PBMC	3/9 (33)	100

*Statistically significant ($P < 0{\cdot}05$) compared with the relevant untreated PBMC (shown in the table).

$P < 0{\cdot}05$). Tumours arose in 6 out of 15 (40%) mice receiving CD45RA-depleted PBMCs, whereas 19 out of 24 (79%) control mice developed tumours (chi-squared with Yates' correction, $P < 0{\cdot}05$). Tumours developed in 3 out of 12 (25%) mice receiving CD45RO-depleted PBMCs, whereas 17 out of 21 (81%) control mice developed lesions (Fisher's exact test, $P < 0{\cdot}005$). Analysis of the median time to tumour did not show any statistically significant difference between test and control groups (Wilcoxon rank sum test, all P-values $> 0{\cdot}1$). The results are summarized in Table II.

These results, together with the data from the analysis of the role of T-cell activation in tumour incidence, suggested that T-cell-derived soluble growth factors were required for tumour outgrowth. As all tumours tested contained very few T cells, we postulated that T cells are essential for the initial outgrowth of tumorigenic EBV +ve B cells, but later become independent of T-cell help and sustain their own growth in an autocrine, cytokine-stimulated manner. This was investigated by analysing human cytokine gene expression of the tumours using RT-PCR.

Analysis of human cytokine gene expression in EBV +ve tumours arising in SCID mice.

Tumours were analysed for the presence of amplifiable human IL-2, IL-4, IL-5, IL-6, IL-10, and IFN-γ, mRNA by RT-PCR. RNA was extracted from 12 peritoneal tumours which had arisen from unfractionated PBMCs from 12 seropositive donors (five high, six intermediate, and one low incidence donors). On two occasions cDNA was prepared from the tumour RNA, and each of the two batches of cDNA were then amplified in a RT-PCR reaction. cDNA from PHA-treated human PBMCs was used as a positive control template, whereas cDNA from PHA-treated murine BALB/c splenocytes was included as a species-specific negative control template. The pattern of cytokine gene expression in the PBMC-derived SCID mouse tumours was compared with that observed in four BLCLs. Amplifiable cDNA was detected in all of the samples tested, as determined by the β-actin RT-PCR. The IL-5 transcript was not detected in any of the test samples. Four out of four (100%) BLCLs expressed human IL-4, IL-6, IL-10 and IFN-γ, but none expressed IL-2. Twelve out of twelve (100%) tumours expressed human IL-10 and IFN-γ, and 11 out of 12 (92%) tumours expressed IL-6. Nine (75%) and eight (67%) out of 12 tumours expressed IL-2 and IL-4, respectively. A representative result showing amplification of human IL-2 and IL-10 is shown in Fig 1. The results are summarized in Table IV. Thus, the main difference observed between BLCLs and the PBMC-derived tumours was the expression of IL-2 in the majority of tumours but in none of the BLCLs. These data indicate that the PBMC-derived SCID mouse tumours have a similar cytokine profile to activated T cells which includes the expression of a variety of B-cell growth factors.

In order to identify the cellular origin of the cytokine transcripts, *in situ* hybridization studies were carried out on serial sections using oligonucleotide and EBER probes. Seven of the ten tumours examined expressed high levels of β-actin and these were further analysed for the expression of human IL-2, IL-4, IL-6, IL-10 and IFN-γ. 100% of the tumours were positive for EBERs, IL-2 and INFγ, whereas 86% were IL-6 and IL-10 positive. 71% of the tumours were IL-4 positive. Representative results showing ISH for IL-2, IL-10 and EBER are shown in Fig 2. The results are summarized in Table V. These results correspond with the RT-PCR data and show that the EBER +ve tumour cells express the cytokines examined.

DISCUSSION

EBV-associated BLPD in transplant recipients carries a mortality rate of up to 70% (Armitage *et al*, 1991). Reduction or withdrawal of immunosuppression, together with the anti-viral agent acyclovir, is currently the treatment of choice, but an optimal therapeutic strategy has yet to be formalized. A better understanding of the pathogenesis of BLPD would help in this formulation of new therapeutic regimes. However, only limited amounts of BLPD biopsy material are available, and this has proved to be very difficult to grow *in vitro*. Recently, BLPD material has been successfully grown directly in SCID mice, but further expansion of this material was not achieved (Perera *et al*, 1996; our unpublished observations). As a result of this, the

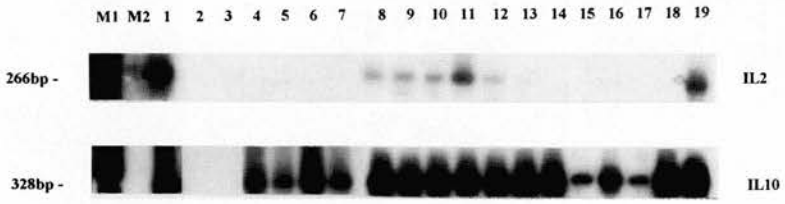

Fig 1. Representative results of the amplification of human cytokine gene transcripts in the tumour material: interleukin 2 (IL-2) and interleukin 10 (IL-10). cDNA from 5 μg of RNA was amplified in a 35 (IL-10)- or 40 (IL-2)-cycle RT-PCR reaction using sequence specific primers in a 100-μl reaction mix. Twenty microlitres of RT-PCR product was run onto a 2·5% w/v NuSieve agarose gel, Southern transferred onto a nylon membrane, and hybridized using a gene-specific ^{32}P-labelled oligonucleotide probe. *Hin*fI-digested φX174 DNA and 1 kbp DNA ladder were used as ^{32}P-radiolabelled and non-radiolabelled DNA size markers respectively. Lane 1, PHA-treated human PBMC; Lane 2, PHA-treated Balb/c murine splenocytes; Lane 3, sterile distilled water; Lanes 4–7, BLCLs A, B, C and D, respectively; Lanes 8–19, human PBMC-derived SCID mouse tumours 1–12 respectively. The size of the IL-2 (266 bp) and IL-10 (328 bp) RT-PCR products is indicated.

Table IV. Data from RT-PCR analysis of human cytokine gene expression in 4 B lymphoblastoid cell lines (BLCLs) and 12 SCID mouse-grown tumours.

		β-Actin	IL-2	IL-4	IL-5	IL-6	IL-10	IFN-γ
Controls	Hu-PBMCs*	+	+	+	+	+	+	+
	Mo-splenocytes†	+	–	–	–	–	–	–
	SDW	–	–	–	–	–	–	–
BLCLs	A	+	–	+	–	+	+	+
	B	+	–	+	–	+	+	+
	C	+	–	+	–	+	+	+
	D	+	–	+	–	+	+	+
Tumours	1§	+	+	+	–	+	+	+
	2§	+	+	–	–	+	+	+
	3¶	+	+	+	–	+	+	+
	4‡	+	+	+	–	+	+	+
	5‡	+	+	+	–	+	+	+
	6§	+	+	+	–	+	+	+
	7§	+	+	+	–	+	+	+
	8‡	+	–	–	–	+	+	+
	9‡	+	+	–	–	+	+	+
	10‡	+	–	–	–	–	+	+
	11§	+	–	+	–	+	+	+
	12§	+	+	+	–	+	+	+

* Human PBMCs treated with PHA *in vitro* for 24 h.
†Murine BALB/c splenocytes treated with PHA *in vitro* for 24 h.
Tumours derived from PBMCs from ‡high, §intermediate or ¶low incidence donors.
+, Positive signal; –,no signal; BLCLs, B lymphoblastoid cell lines; SDW, sterile distilled water.

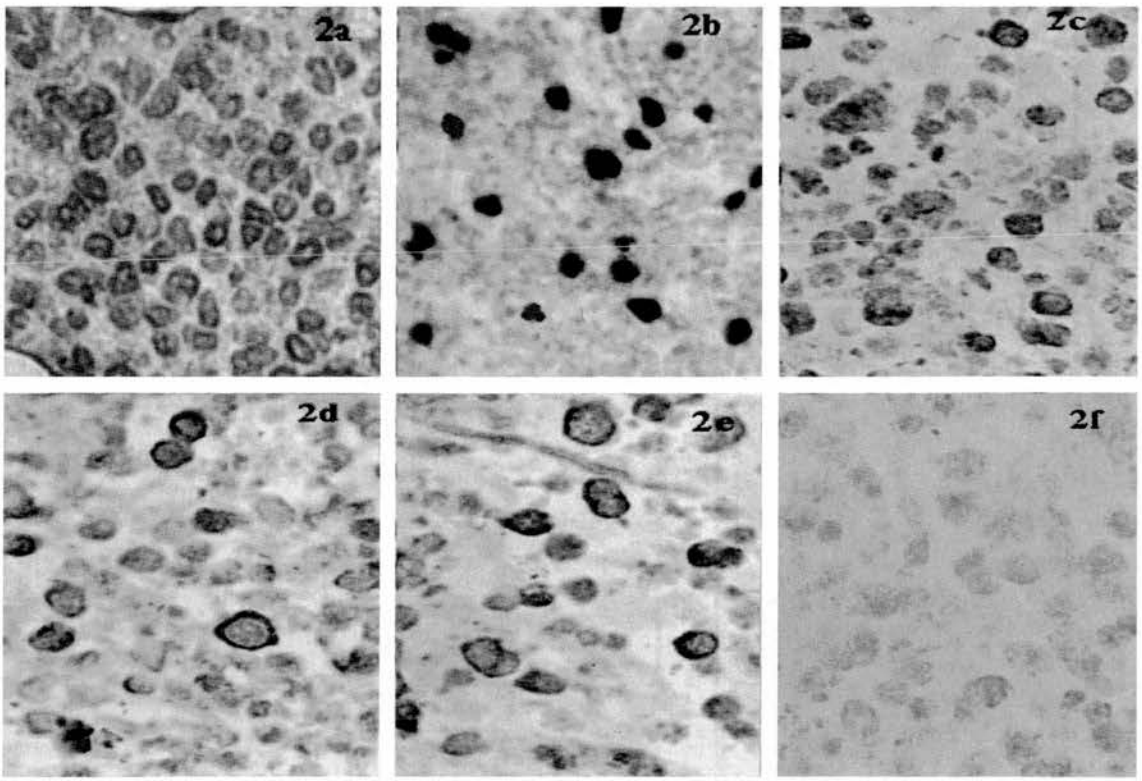

Fig 2. Representative results of *in situ* hybridization for EBV-encoded RNAs (EBERs) and human cytokine gene transcripts: interleukin-2 (IL-2) and interleukin-10 (IL-10). Serial sections of tumour 2 with B-cell-specific anti-CD20 mAb (Fig 2a), EBER probe (Fig 2b), β-actin probe (positive control) (Fig 2c), IL-2 probe (Fig 2d), IL-10 probe (Fig 2e), no probe control (Fig 2f). (original magnification ×400).

Table V. Data from *in situ* hybridization analysis of human cytokine gene expression in 10 SCID mouse-grown tumours.

		β-Actin	IL-2	IL-4	IL-6	IL-10	IFN-γ
Controls	Hu-PBMCs*	+	+	+	+	+	+
	No probe	–	–	–	–	–	–
	RNAase treatment	–	–	–	–	–	–
	Mo tissue	NT	–	–	–	–	–
Tumours	1‡	4 +	4 +	3 +	3 +	1 +	4 +
	2‡	4 +	4 +	–	4 +	4 +	4 +
	5†	2 +	3 +	2 +	–	1 +	1 +
	6‡	5 +	3 +	–	3 +	4 +	3 +
	7‡	2 +	2 +	2 +	2 +	2 +	NT
	8†	3 +	1 +	1 +	3 +	–	2 +
	9†	1 +	–	–	–	1 +	–
	10†	3 +	2 +	2 +	1 +	3 +	3 +
	11‡	1 +	–	NT	–	–	–
	12‡	1 +	–	–	–	–	–

*Human PBMCs treated with PHA *in vitro* for 24 h.
Tumours derived from PBMCs from †high or ‡intermediate incidence donors.
Percentage of positive cells: 5+, > 80%; 4+, 51–80%; 3+, 21–50%; 2+, 6–20%; 1+, 1–5%; –, 0%; NT, not tested.

present study was designed to investigate the pathogenesis of EBV-associated BLPD using the PBMC-inoculated SCID mouse model (Mosier *et al*, 1988).

Our experimental design was to separate PBMCs into CD2 +ve T-cell and non-T-cell populations and then deplete the T cells of specific subpopulations. The depleted fractions were then recombined with the non-T cells prior to inoculation into mice. Tumour incidence in these mice was compared with tumour formation in animals receiving unseparated PBMCs. The use of tumour outgrowth in mice inoculated with unfractionated PBMCs as a control, rather than in animals inoculated with recombined B and T cells, could possibly have exaggerated differences in tumour formation between control and test mice. However, we did not find a significant difference in tumour incidence when carrying out initial studies comparing tumour outgrowth in mice inoculated with either unfractionated or fractionated and recombined PBMCs (Table II).

The inoculation of T-cell-depleted PBMCs significantly reduced the tumour incidence, indicating that T cells were required for tumour formation despite the B-cell origin of BLPD. This result is in line with earlier observations (Veronese *et al*, 1992). Removal of CD4 +ve T cells from PBMCs prior to inoculation gave a highly significant reduction in tumour incidence, and removal of CD8 +ve, CD45RO +ve and CD45RA +ve T cells also resulted in a significant, but less dramatic, reduction. As either CD45RA or CD45RO antigens are expressed on both CD4 +ve and CD8 +ve T-cell subsets (Thomas, 1989), the reduction of tumour incidence following their depletion probably reflects a reduction in numbers of both the CD4 +ve and CD8 +ve T-cell subsets in the inoculum. Our results contrast with those of Veronese *et al* (1992) who found only a minor, non-significant, reduction in tumour incidence in mice inoculated with CD4-depleted PBMCs, and a minor increase in tumour incidence when mice were inoculated with CD8-depleted PBMCs. In their study, Veronese *et al* (1992) inoculated 60×10^6 to 100×10^6 unfractionated or fractionated PBMCs into each SCID mouse, whereas we inoculated 42×10^6 to 54×10^6 unfractionated PBMCs into each control animal and depleted that same number of cells of a specific T-cell subset before injection into a test mouse. Furthermore, Veronesi *et al* (1994) obtained tumour outgrowth when B cells were inoculated into SCID mice depleted of murine NK cell activity with an anti-asialo GM1 antiserum. We did not find the administration of this antiserum to have a significant effect on tumour outgrowth in PBMC-inoculated SCID mice (data not shown) and we did not therefore routinely use it in our animals. Thus, it is difficult to compare the results obtained between the two laboratories due to variability in engraftment and lymphoma development, and lack of a consensus protocol.

In order to investigate the nature of the T-cell assistance required for tumour development, we stimulated T-cell cytokine production with PHA *in vitro* prior to inoculation into SCID mice, and demonstrated a significant decrease in the median time to tumour development. Taken in conjunction with the finding of a reduction in tumour incidence following removal of CD30 +ve activated T cells, this suggests that activated T cells are involved in promoting tumour growth. These results cannot be attributed to elimination of CD30 +ve B lymphocytes (and thus EBV-infected cells) from the test inoculum as only E-rosette-positive populations were depleted of CD30 cells. Thus, in the depletion experiments, all animals (whether control or test mice) received similar numbers of B cells.

We postulated that the role of T cells in tumour outgrowth is to supply the tumorigenic EBV +ve B cells with essential growth factors. Direct cell-to-cell contact may also be of importance, although antibody stimulation of the CD40 co-stimulatory molecule on B cells has been shown to inhibit

Fig 3. Model of tumour outgrowth in the SCID mouse.

(rather than promote) lymphomagenesis in the PBMC-injected SCID mouse (Murphy *et al*, 1995; Funakoshi *et al*, 1997). We used RT-PCR to analyse the human growth factors involved in tumour formation. 100% of tumours tested expressed mRNA for human IL-10 and IFN-γ, and the majority also expressed IL-2, IL-4 and IL-6 mRNA. All of these cytokines are potential stimulators of B-cell growth. As very few T cells were demonstrated in the tumours, it seemed likely that the cytokine mRNAs were transcribed by the tumour cells. To confirm this suspicion, *in situ* hybridization studies were carried out to determine the cellular origin of the cytokine transcripts. Analysis of serial sections of the tumours probed with EBER and cytokine probes confirmed the RT-PCR results and showed that the tumour cells themselves expressed the cytokines tested (Fig 2).

Our results confirm and extend the observations by Baiocchi *et al* (1995), who demonstrated the expression of human IL-10 and its receptor in PBMC-derived SCID tumours. Based on their results, and the observation that human IL-6 or IL-10 maintain *in vitro* viability of fresh SCID tumour material, the group suggested that human IL-6 and IL-10 may be involved in an autocrine stimulatory loop favouring tumour outgrowth in the SCID mouse (Khatri *et al*, 1997).

Our results suggest that in the PBMC-inoculated SCID mouse the sequence of events leading to tumour formation is as follows: (1) T cells mediate initiation of outgrowth of EBV +ve tumorigenic B cells; (2) maintenance of tumour growth in an autocrine, cytokine-stimulated manner occurs; (3) tumour formation becomes independent of T-cell assistance (Fig 3). This model is supported by our recent finding that T-cell-rich primary BLPD biopsy material gives rise to biopsy-derived tumours in the SCID mouse model (unpublished observations). These biopsy-derived SCID tumours show paucity of T cells but express (as do the primary lesions) human IL-2, IL-6, IL-10 and IFN-γ similar to that observed in this study in the PBMC-derived SCID tumours. Therefore, similar mechanisms may lead to BLPD in the SCID mouse and in the graft recipient, thus making the murine model a valuable tool in which to investigate the disease and test novel therapeutic regimens against BLPD in the organ transplant patient.

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